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PRINCIPAL INVESTIGATOR: Margaret C. Neville, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Aurora, CO 80045-0508

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**6. AUTHOR(S)**
Margaret C. Neville, Ph.D

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Colorado Health
Sciences Center
Aurora, CO 80045-0508

E-Mail: peggy.Neville@uchsc.edu

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**13. ABSTRACT (Maximum 200 Words)**

We have fully characterized the expression and localization of claudin 7 in the mammary epithelium, finding that it is not associated with tight junctions but is constitutively present in punctate structures, presumably vesicles, at the basal and lateral borders of cells. The same is true of bronchiole and certain elements of the renal epithelium while claudin 7 is found associated with tight junctions of the epididymus and mouse mammary cell lines. Four types of murine mammary tumors were examined. In all claudin 7 showed a distinct cytosolic and basolateral distribution. We find claudins 1, 3, 4, 5, 7 and 8 to be present in at least one of our mouse mammary cell lines, where they are localized to the tight junction. Only claudin 1 has been found to be consistently present in mammary epithelial tight junctions. Claudin 3 is localized to the basal and lateral borders of the cells in the virgin and pregnant animals, but not observed in lactation. Claudin 8 is prominent at the tight junctions in the mammary epithelium from the lactating animal. One paper has been submitted detailing these findings and a second is in the final stages of preparation.

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## INTRODUCTION

The purpose of the research funded under the above research grant was to examine the hypothesis that the gene for the tight junction protein, claudin-7, plays a role in breast cancer. We planned to test the hypothesis that high expression of claudin-7 can signify one or more novel roles in tumorigenesis by:

1. Determining the cellular and subcellular localization of claudin-7 in the mouse mammary gland.
2. Comparing mRNA levels of claudin-7 to claudin-1, claudin-3 and two adherens proteins, E-cadherin and beta-catenin.
3. Examining the functional significance of claudin-7 by overexpressing it in cell lines and in the mammary gland.

Objective 1, which comprises TASKS 1, 2 and 3 in the statement of work was completed in the first year. In the intervening two years we refined our immunocytochemical analysis to show that claudin 7 is not only a constitutive component of the mammary luminal epithelium but is present solely at the basal-lateral borders of the cell and does not overlap the tight junctions. It appears both to be present in the membranes and in vesicles near the membrane. These data have been written up for publication and a .pdf of the submitted document is attached.
Objective 2, which comprises parts of tasks 5 and 6 was largely completed based on microarray analysis last year. In addition we examined claudins 1-6 and 8 in more detail, using antibodies to obtain localization of these molecules. Details of the localization of some of these claudins will be presented in this report
Objective 3 was changed to examine the functional significance of claudins. Because we have found significant changes in claudins 3 and 8, we have cloned these molecules and plan to use them in expression studies in the normal gland. This task is still ongoing.

We spent much time determining the specificity of the available claudin antibodies and have found this to be a particularly difficult task, as many of the antibodies available, particularly those from Santa Cruz, do not work as advertised. The criteria we use for antibody specificity are:

1. The antibody shows localization to the tissue in which it has been demonstrated by previous investigators and does not stain tissues that have been shown to be negative by immunohistochemistry or Northern analysis.

2. Localization to tight junctions by immunohistochemistry in mouse mammary cell lines that form tight junctions in vivo, either EPH4 cells or CIT3 cells.

3. Blocking by incubation with the specific peptide against which the antibody has been made.

We will start the report by summarizing the results of our studies on claudin-7 and then summarize our progress with the other claudins in the 1 to 8 series.

## BODY:

### 1. Claudin-7 localization in the mammary epithelial cell.

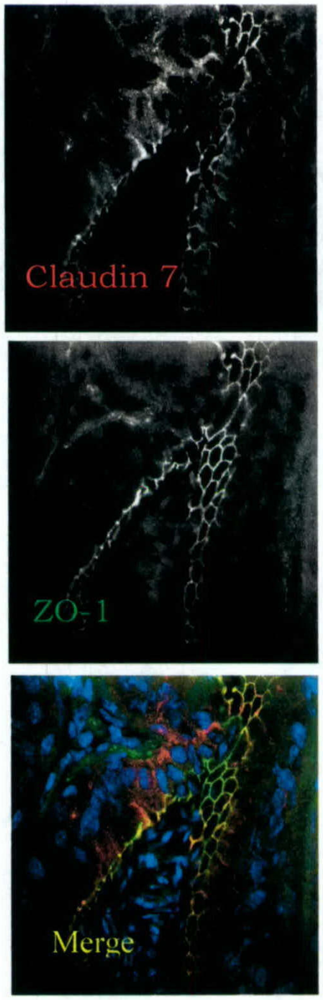


Figure 1. Mouse epididymis stained with antibodies to claudin 7 and the tight junction protein ZO-1. Note the overlap between Claudin 7 and ZO1 staining in the merged image (Yellow).

Our PCR studied showed that claudin-7 is a constitutive component of mammary epithelial cells at all stages of mammary development and that it is also present in mammary tumors. We had an antibody made by Zymed and showed that it cross reacts with the protein on Western blots (TASK 4), stains only the luminal epithelium in sections of mouse mammary gland, stains tight junctions in EPH4 cells (Figure 5) and reacts with bronchiole epithelial cells, accounting for detection of the mRNA in the lung (1) but not with cells in the liver where it is not present by mRNA analysis. The antibody stains both frozen and paraffin sections. Staining is blocked by the peptide against which the antibody was made and was not seen in the absence of antibody. All these criteria indicate that the antibody is specific for claudin-7. This year we have additional data showing that the protein does localize to tight junctions the epididymus (Figure 1). Note that the stain in the upper panel coincides at least in part with the cobblestone stain in the second panel indicating overlap at the tight junctions in this tissue, where there also appears to be cytoplasmic stain. However, Claudin 7 appears to be completely excluded from the tight junctions of mammary epithelial cells as shown by the data in Figures 2 and 3. The higher power view in Figure 3 shows that the stain is punctate in nature suggesting a vesicular localization near the borders of the cell. This significance of this localization is not known at this point. It is however, one of very few documented instances where the claudin doesn't localize to the tight junction.

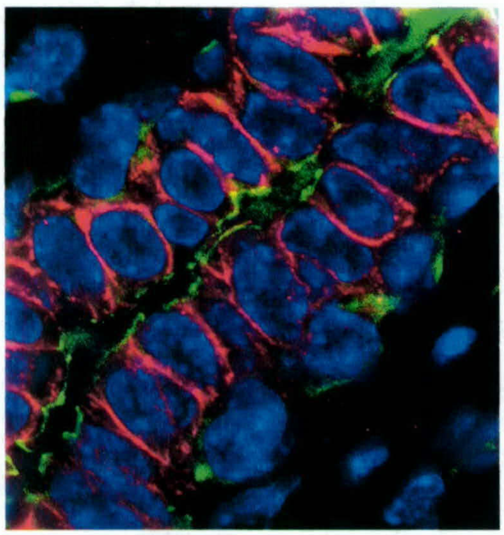

Figure 2. Section of a mammary gland from a pregnant mouse showing complete segregation of green (ZO-1) and red (claudin 7) stain. The nuclei are stained blue with DAPI.

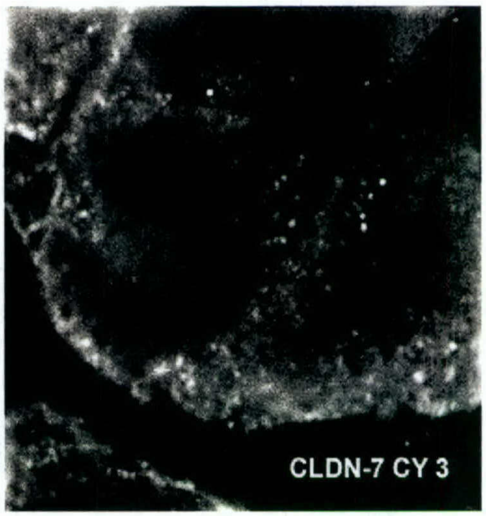


Figure 3. High power section of the basal portion of an epithelial cell from a lactating gland showing the punctate nature of the claudin-7 stain.

**2. Claudin expression in the mammary epithelium—Real time PCR, in situ and microarray analysis.**

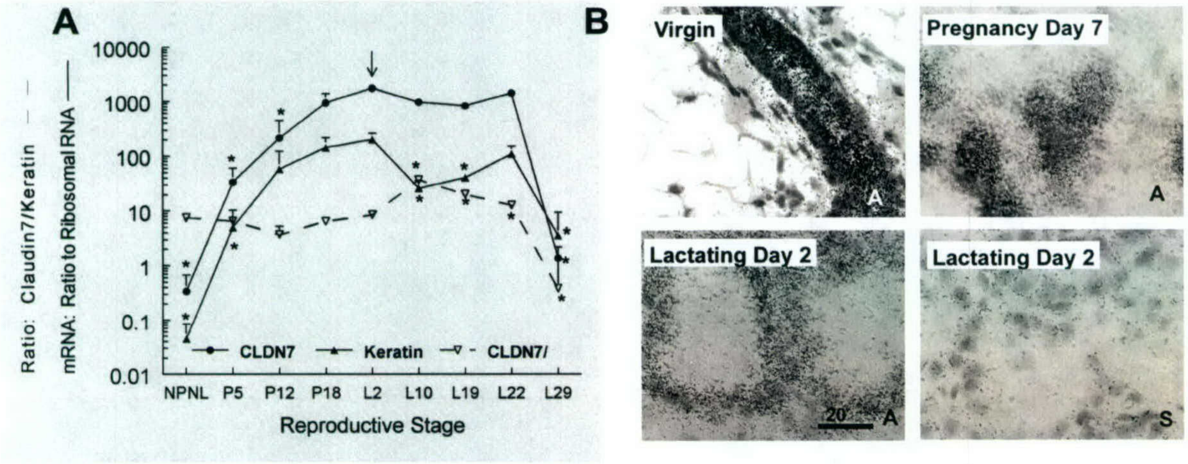


**Figure 4. Developmental expression of claudin 7 mRNA in the murine mammary gland.** A. Real time RT-PCR measurement of claudin 7 and keratin 19 from total mammary glands of non-pregnant non-lactating (NPNL) mice, mice at 5, 12, and 18 days of pregnancy (P5, P12, P18) and 2, 10, 19, 22 and 29 days post partum (L2, L10, L19, L22, L29). By L29 the pups have weaned themselves and mammary gland involution is nearly complete. Dotted line: Ratio of claudin-7 RNA to Keratin 19 at the same time points. Three mice analyzed at each time point. Bars are ± 1 s.e.m. Where no bar is apparent, the standard deviation falls within the symbol. Asterisks indicate points that differ significantly from values at L2, $p < 0.05$. B. *In situ* hybridization of claudin 7 probes to sections from virgin, pregnant and lactating mammary glands. Sections labeled A were hybridized to the antisense probe; the lower right hand section labeled S is the sense control. Bar is 20 microns.

We used real time PCR to examine the expression of claudin 7, comparing its expression to that of keratin 19, a marker for mammary epithelial cells. Its expression paralleled that of keratin 19 throughout the developmental cycle of the mammary gland (Figure 4), suggesting that the molecule is a constitutive component of the mammary epithelial cell. This hypothesis was confirmed by in situ hybridization analysis where the RNA was found associated only with the mammary epithelium. We then expanded our

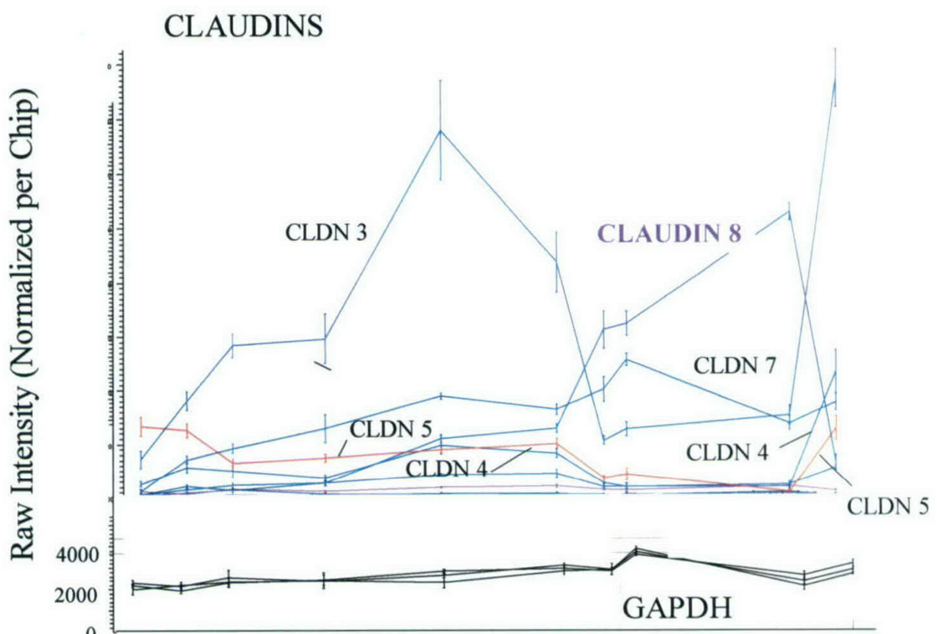


Figure 5. Microarray data for developmental regulation of claudin expression in the mammary gland from the virgin (V) through pregnancy days 1, 3, 7, 12 and 17, lactation days 1, 2 and 9 and two days of forced involution after pup removal at day 9 (I2).

microarray analysis of mammary gland claudins during normal development. Figure 5 shows the expression of claudins 1 through 8 in the mammary epithelium in a developmental series that used 4 animals for every point compared to GAPDH expression. Claudin 3 increases through pregnancy dropping off sharply with the onset of lactation. Claudin 8, on the other hand, is highly expressed during pregnancy. Claudins 4 and 5 also appear to be developmentally regulated. Claudins 1, 2 and 6 are expressed at very low levels and we have, of course, already described the expression of claudin 7 (above and last years progress report). We procured antibodies to all these claudins to determine whether the protein was expressed and its localization. Table 1 and the paragraphs below summarize our progress in this regard.

3. **Immunohistochemical staining for Claudins 1-6 and 8 in the mammary gland.**

Table 1 summarizes our progress to date. At this point we are missing only some controls. The results will by summarized by claudin. For all antibodies we have examined staining in EPH4 and CIT3 lines of mouse mammary epithelial cells. In addition we have generally examined tissues reported to be either positive or negative for the claudin, although this analysis is not complete. We have also examined staining in the mouse mammary gland in virgin, pregnant and lactating animals. Where we found stain associated with the tight junctions we have or will procure a blocking peptide to further test the specificity of the stain. We show only selected results with Claudins 1, 3 and 8.

*Claudin 1.* Although claudin 1 gene expression is low we did find claudin 1 localized to tight junctions at all stages of development. Figure 5 shows results from the virgin mouse, but similar observations were made in pregnancy and lactation. Interestingly the stain is concentrated at the junction but extends beyond the junction in a way that suggests that it may be trafficking to or from the junction in vesicles. This was a common finding in all preparations where claudins were observed at tight junctions.

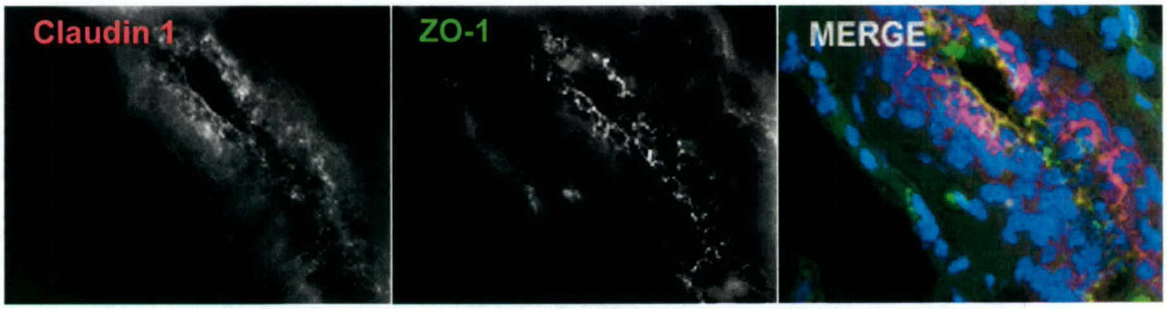


Figure 6. Stain for Claudin 1 (red), ZO1 (green) and nuclei (blue) in a frozen section from mammary duct from a virgin mouse. The lattice work at the apical borders of the cells stains for both claudin 1 and ZO1 as shown clearly in the black and white versions that show each fluor separately.

**Table 1: Immunohistochemistry of claudins 1 through 8 through mammary development**

| | QRT1 | Array | peptide block | Tissue culture | Postive control | Negative control | Virgin | Pregnancy | Lactation |
|---|---|---|---|---|---|---|---|---|---|
| **Claudin 1** | TBD | present, low | YES, OK | YES | mammary gland | Blood vessels | All stages--colocalizes with ZO1 at tight junctions | | |
| **Claudin 2** | TBD | Absent, very low | NO | Not Seen | Renal control TBD | EPH4 cells; Lung | No expression at TJ | | |
| **Claudin 3** | Down at lac | Down at Lac | No peptide available | YES, EPH-4 at junctions | TBD | TBD | Not at junctions, punctate in cytoplasm | | None |
| **Claudin 4** | Down at lac | Present preg; Absent lactation; high involution | NO | CIT3-yes | EPH4--yes | TBD | Nothing at TJ, but possible interstitial stain (blood vessels?) in lactating gland | | |
| **Claudin 5** | TBD | Present preg; Absent lactation; high involution | NO | CIT3 yes, EPH4, yes | TBD | NO | Vague diffuse apical stain; interstitial stain | | |
| **Claudin 6** | TBD | Absent | | No stain | TBD | NO | Background only | | |
| **Claudin 7** | Yes | Expression constant | Yes | Yes | Epididymis | Liver | Expressed at all stages, but localized basally and laterally | | |
| **Claudin 8** | Yes | Up at Lac | Yes | no-EPH4; yes;CIT3 | Kidney | Heart-no stain | Not at TJ | Apical, some TJ, not all | Clear stain at tight junctions |

TBD, to be done; Lac, lactation; Preg, pregnant; EPH4 and CIT3, mouse mammary cell lines; TJ, tight junction

*Claudin 2.* We were unable to find stain for claudin 2 in any of the mammary tissues or cell lines observed. We have not yet verified the positive control for this claudin so it may be that the antibody is not working on our sections.

*Claudin 3.* Claudin 3 gene expression increases markedly at mid pregnancy with a sharp decline at the onset of lactation. Although claudin 3 stained tight junctions in EPH4 cells (data not shown) it was distributed at the basal and lateral borders of mammary epithelial cells from virgin and pregnant mice. Figure 7 shows that this claudin is distributed at tight junctions in EPH4 cells suggesting that the antibody does indeed stain for a claudin. Figure 8 shows a paraffin section of a mammary gland from a pregnant mouse. ZO1 (green) shows a clear lattice work at the apical borders of the cell but claudin 3 stain (red) does not overlap with ZO-1 but is confined to the cytoplasm. No claudin 3 stain was observed in lactating mice. The implications of its cytosolic expression in pregancy are not clear. It will be of interest to determine whether this pattern is repeated in mammary tumors as it is for claudin 7.

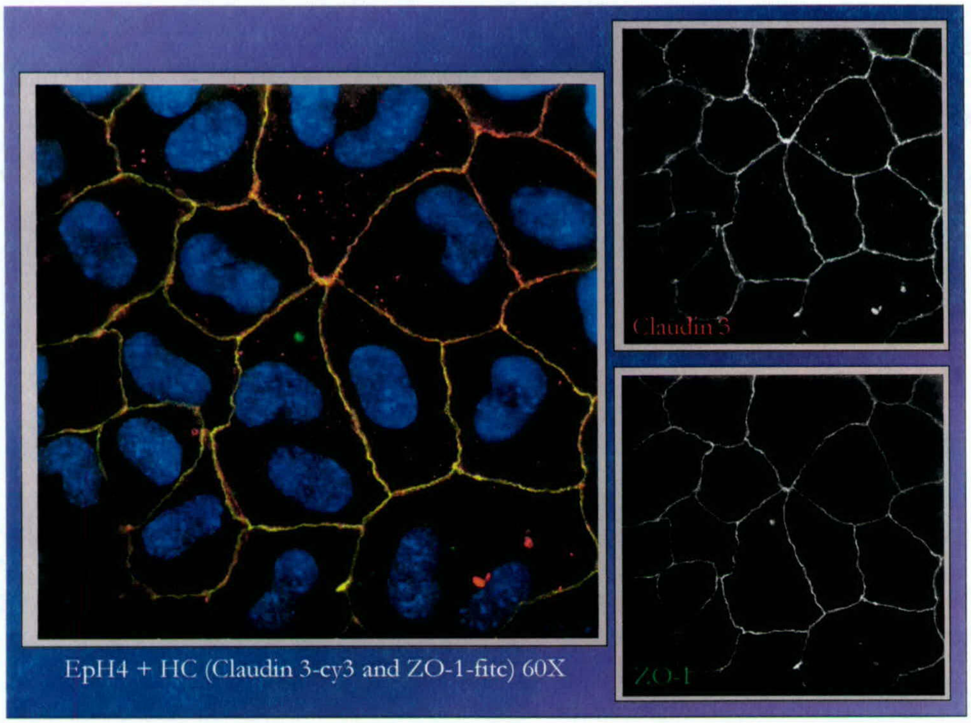


Figure 7. Claudin 3 stain in EPH4 cells. Claudin 3 (red) and ZO1 (green) are merged in this image from of cultured cells taken with a digital confocal microscope.

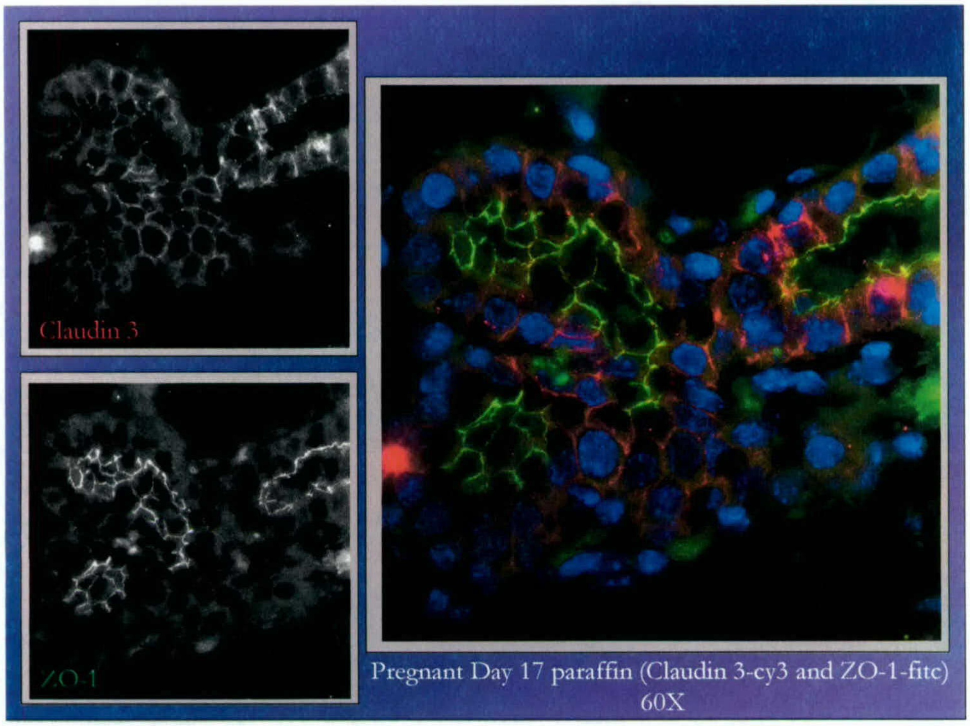


Figure 8. Claudin 3 and ZO1 staining of a paraffin section from a mammary gland of a pregnant mouse. Stains for the two probes do not overlap. In addition Claudin 3 stain is excluded from the nucleus.

*Claudin 4.* From the array data, claudin 4 was present at pregnancy but absent at lactation, increasing again at involution in mammary tissue. This finding has been confirmed by real time PCR. The antibody showed positive staining at tight junctions in both EPH4 and CIT3 cells, However, there was no stain in the mammary epithelium although there appeared to be some interstitial stain which could represent blood vessels.

*Claudin 5.* Gene expression was low in pregnancy and lactation but increased at involution. Tight junction stain was observed in both EPH4 and CIT3 cells, but no stain was observed in either the pregnant or lactating gland. We have not yet examined involution.

*Claudin 6.* Gene expression was very low at all stages of mammary development and we were unable to detect any stain in either cultured cells or mammary epithelium. The positive control has yet to be done for this claudin and the antibody may be suspect because it came from Santa Cruz. The remainder of our antibodies are from Zymed, a company we have found to be more reliable

*Claudin 8.* Claudin 8 is the most interesting of the claudins examined to this point. Its gene expression is low through pregnancy with a sharp increase at lactation. We detected the protein, using a purpose made antibody from Zymed, at the tight junctions of CIT3 cells but not in EPH4 cells. We did not detect stain in virgin tissue. Occasional tight junctions were stained in the pregnant animal, whereas stain was consistently observed at tight junctions in the lactating animal (Figure 8). This finding is exciting because claudin 8 has been associated with reduced permeability in cultures of MDCK cells (1). We have previously reported that claudin 8 mRNA is present in mammary tumors, but we have not yet examined these tumors for the presence and localization of the protein.

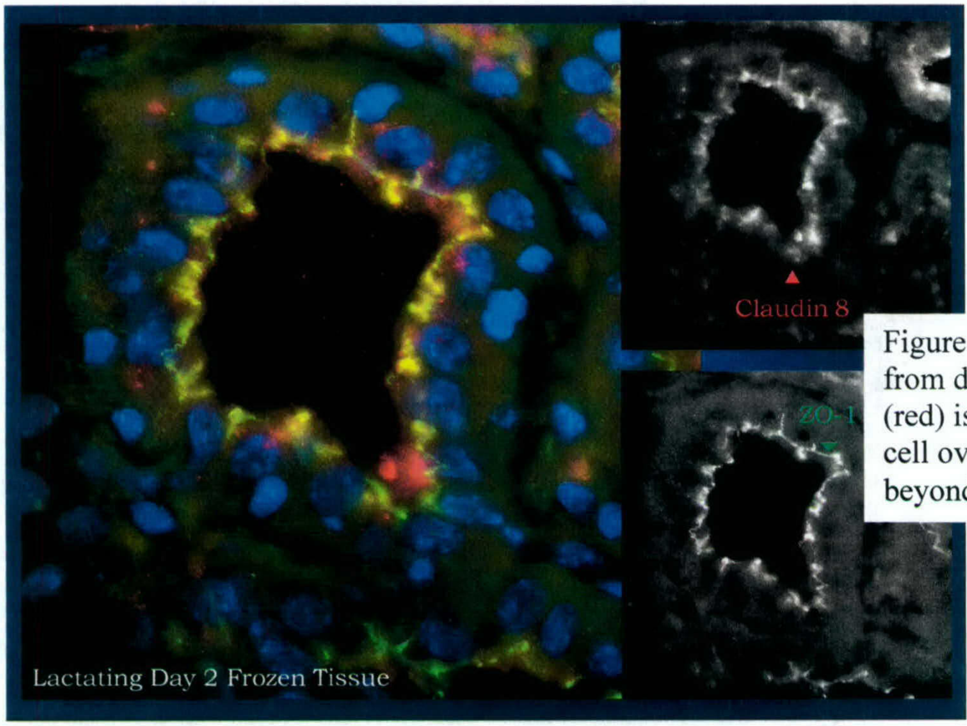


Figure 8. Claudin 8 staining in a section from day 2 lactating tissue. Claudin 8 (red) is found at the apical borders of the cell overlapping with and extending beyond ZO-1 stain.

KEY RESEARCH ACCOMPLISHMENTS since the beginning of the grant.

- A specific and highly active antibody to claudin 7 has been developed and shown to react with claudin 7 on Western blots (tasks 1and 4).
- Claudin-7 is localized to luminal epithelial cells in the mammary gland throughout development by *in situ* hybridization and specifically to the basolateral membranes of these cells in both normal glands and mammary tumors as shown by immunohistochemistry (Task 2). This claudin is present at tight junctions in cultured cells and epidymus, but not in lung or mammary epithelium. A submitted paper detailing our findings with claudin 7 has been submitted (see appendix)
- The developmental expression of claudin-7 mRNA was shown to parallel expression of keratin by real time RT-PCR and high expression was found in a series of tumors (Task 3).
- Expression of additional claudins and claudin-like molecules has been quantitated by microarray analysis using Affymetrix arrays (Task 5). The expression of claudin 8 has been confirmed by real time RT-PCR and found to increase more than 10 fold at lactation. The protein was found in the tight junctions of the mammary epithelium only in the lactating gland.
- Claudin 8 is expressed in mammary tumors although they lack tight junctions. (Task 5).
- Claudins 1 has been localized to tight junctions in mammary epithelial tissues throughout development (Task 5).
- Claudins 3, 4, 5, and 8 were observed at tight junctions in mammary cell lines. However, claudin 8 was the only one of these associated with the mammary tissue tight junction, and then only at lactation.
- Claudins 2 and 6 were not observed in any mammary epithelium.

## REPORTABLE OUTCOMES.

A paper describing the use of adenoviral transduction to express foreign genes in the mammary epithelium has been published in J. Virol. (Appendix). This study was started with the support of a previous DOD breast cancer grant and was completed in the project period of the present grant.

A paper describing our findings with claudin 7 has been submitted (Manuscript in appendix)

A paper describing the expression and localization of claudins during mammary development will be submitted to J. Physiol.

A poster detailing our findings with claudin 7 was presented at the Era of Hope meeting in Orlando, Fl in October, 2002. (Abstract in Appendix)

A poster detailing our findings with Claudin 7 was presented at the Sacramento Breast Cancer meeting in October, 2003 (abstract in appendix)

A poster describing our findings with mammary gland claudins was presented at the Society for Gynecological Investigation in March, 2004 (abstract in appendix)

## CONCLUSIONS.

We have now fully characterized the expression and localization of claudin 7 in the mammary epithelium, finding that it is not associated with tight junctions but is constitutively present in punctate structures, presumably vesicles, at the basal and lateral borders of cells. We find the same to be true of mouse mammary tumors. We are now in the process of fully characterizing the expression of claudins 1 through 6 and claudin 8 in the developing mammary epithelium and in mammary cell lines. We find claudins 1, 3, 4, 5 and 8 to be present in at least one of our mouse mammary cell lines, where they are localized to the tight junction. However, only claudin 1 has been found to be consistently present in mammary epithelial tight junctions. Claudin 3 is localized to the basal and lateral borders of the cells in the virgin and pregnant animals, but not observed in lactation. Claudin 8 is prominent at the tight junctions in the mammary epithelium from the lactating animal but not at other developmental stages suggesting it may participate in the closure of tight junctions observed during lactation.

These studies are important because they set the stage for more detailed examination of the function of the claudins in both the normal mammary gland through development and breast tumors. The finding of the basolateral localization of claudin 7 is particularly intriguing for it may be involved with interactions of vesicular components of the cells with basolateral elements and may contribute to stable cell-stroma interactions.

**Key personnel associated with the Project**

Margaret C. Neville, Ph.D. P.I.
Brigitte Blackman, Ph.D. Post-doctoral fellow
Steven Nordeen, Ph.D. Consultant
Julia Foo, PRA
Valerie Burns, PRA
Michael Rudolph, PRA
William Zabaronick, PRA
Tanya Russell, PRA
Neal Beeman, Graduate student

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1. Yu AS, Enck AH, Lencer WI, Schneeberger EE. Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation. J. Biol. Chem. 278: 17350-9, 2003.

APPENDICES

**1.** Tanya D. Russell, Neal E. Beeman, Emily F. Freed, Margaret C. Neville, Jerome Schaack. (2003) Transduction of the Mouse Mammary Epithelium with Adenoviral Vectors *in vivo*. *J. Virol.* 2003;77:5801-9.

**2.** Brigitte Blackman, Tanya Russell, Steven K. Nordeen, Daniel Medina, and Margaret C. Neville , Claudin-7 is not just a tight junction protein: Expression and localization in the normal murine mammary gland and murine mammary tumors. Submitted to Breast Cancer Research.

**Abstracts**

**3.** Margaret C. Neville, Brigitte Blackman, Julia Foo, Valerie Sawicki. EXPRESSION OF CLAUDIN 7 IN THE MOUSE MAMMARY EPITHELIUM, ERA OF HOPE, Fall, 2002

4. Margaret C. Neville, Brigitte Blackman, Julia Foo, Valerie Burns, Neal Beeman. Expression of Claudin 7 in the mouse mammary epithelium and tumor models. Breast Cancer Research Meeting, Sacramento, CA, October 2003

5. Margaret C. Neville, Ph.D. , Brigitte Blackman, Ph.D. , Julia Foo , Valerie Burns and Neal Beeman, M.S EXPRESSION AND LOCALIZATION OF THE CLAUDINS IN MOUSE MAMMARY GLAND AND TUMOR MODELS, SGI, Spring, 2004

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# Transduction of the Mammary Epithelium with Adenovirus Vectors In Vivo

Tanya D. Russell,[1] Andreas Fischer,[1] Neal E. Beeman,[1] Emily F. Freed,[1] Margaret C. Neville,[1] and Jerome Schaack[2]*

*Department of Physiology and Biophysics[1] and Department of Microbiology,[2] University of Colorado Health Sciences Center, Denver, Colorado 80262*

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**Because the mammary parenchyma is accessible from the exterior of an animal through the mammary duct, adenovirus transduction holds promise for the short-term delivery of genes to the mammary epithelium for both research and therapeutic purposes. To optimize the procedure and evaluate its efficacy, an adenovirus vector (human adenovirus type 5) encoding a green fluorescent protein (GFP) reporter and deleted of E1 and E3 was injected intraductally into the mouse mammary gland. We evaluated induction of inflammation (by intraductal injection of [$^{14}$C]sucrose and histological examination), efficiency of transduction, and maintenance of normal function in transduced cells. We found that transduction of the total epithelium in the proximal portion of the third mammary gland varied from 7% to 25% at a dose of $2 \times 10^6$ PFU of adenovirus injected into day 17 pregnant mice. Transduction was maintained for at least 7 days with minimal inflammatory response; however, significant mastitis was observed 12 days after transduction. Adenovirus transduction could also be used in the virgin animal with little mastitis 3 days after transduction. Transduced mammary epithelial cells maintained normal morphology and function. Our results demonstrate that intraductal injection of adenovirus vectors provides a versatile and noninvasive method of investigating genes of interest in mouse mammary epithelial cells.**

The mammary gland is a compound lobulotubular structure that is a reliable model for developmental studies of cellular growth and differentiation, epithelium-stroma interactions, and tissue-level analysis of systemic hormonal regulation (13). In humans, it begins to develop in the 18- to 19-week fetus and after birth remains quiescent until puberty, when hormonal stimulus by estrogen and growth hormone triggers the tree-like branching of a network of ducts that extends from the nipple into the mammary fat pad on the anterior wall of the thorax (12, 13). Lobular structures, which will become the milk-secreting acini, originate from these ducts. Lobular development is highly dependent upon hormonal stimulation, and in humans, in whom these lobular structures are known as terminal duct lobular units, begins after the onset of menses. In mice the extent of lobular development in the virgin animal is strain dependent (12). Full alveolar development and maturation of the mammary epithelium take place during pregnancy and are dependent upon high circulating concentrations of progesterone, prolactin, and/or placental lactogen (12). Upon withdrawal of progesterone at parturition, lactation commences. Ongoing milk secretion continues during lactation under the influence of prolactin and oxytocin and ceases at weaning. When regular extraction of milk ceases, the alveolar epithelium undergoes apoptosis and remodeling, and the gland reverts to a prepubertal stage (12).

Since the mammary gland undergoes its functional morphogenesis in the young adult to adult stages and is very susceptible to tumorigenesis (13), it would be desirable to be able to manipulate its genetic complement at different developmental stages and study the effects of these changes. For this reason, we investigated the utility of adenovirus transduction in vivo to alter gene expression by using injection through the nipple to gain access to the epithelium from the exterior of the animal. Earlier investigators have taken advantage of intraductal injection techniques, using injections in goats and mice to study the permeability of the mammary epithelium to $Na^+$, $Cl^-$, and radiolabeled sucrose (8, 16).

DEAE-dextran-mediated transfection has been used to obtain human growth hormone expression in the guinea pig mammary gland after intraductal injection (5). However, the proportion of transfected cells was quite low. On the other hand, adenovirus transduction has proven to be a suitable method for efficient transduction of primary mammary cells in vitro in combination with mammary gland reconstitution to yield highly efficient gene transfer (18). In vivo, Jeng and coworkers injected an adenovirus vector coding for β-galactosidase into the rat mammary gland through the mammary duct and obtained significant expression of the gene (6). Yang et al. (24) obtained expression of LacZ in the mouse mammary gland in vivo after intraductal injection of an adenovirus vector. Although these studies demonstrated the effectiveness of adenovirus vectors, the issues of an inflammatory response and the efficiency of transduction have not been fully addressed.

We are primarily interested in the transition from pregnancy to lactation and sought a noninvasive, noninflammatory delivery system for introducing foreign genes into the mammary epithelium without transduction of the surrounding stroma. Our laboratory has perfected a technique of intraductal microinjection into the mouse mammary gland (14) and has used this

* Corresponding author. Mailing address: Department of Microbiology, Box B175, Room 4617, University of Colorado Health Sciences Center, Denver, CO 80262. Phone: (303) 315-6883. Fax: (303) 315-6785. E-mail: jerry.schaack@uchsc.edu.

technique to analyze tight junction regulation (15) and tight junction permeability relative to progesterone withdrawal and the presence of glucocorticoids (16) in the late pregnant mouse. We hypothesized that a similar intraductal injection technique could be used to obtain direct and localized transduction of the mammary epithelium with adenovirus vectors with minimal inflammation and little stress to the animal. The present study demonstrates that intraductal injection of a green fluorescent protein (GFP)-encoding adenovirus vector at late pregnancy leads to successful transduction of the epithelial cells in the proximal portion of the gland that lasts through parturition and into at least 5 days of lactation without inflammation. Although we examined transduction at late pregnancy most carefully, we also present data obtained at other stages of mammary gland development.

## MATERIALS AND METHODS

**Animals.** CD-1 mice aged 5 to 9 weeks were purchased from Charles River Breeding Laboratory (Wilmington, Mass.) and maintained in the U.S. Department of Agriculture-approved Center for Laboratory Animal Care of the University of Colorado Health Sciences Center Animal Care Facility. Nulliparous, early pregnant (3 days; P3), late pregnant (17 days; P17), and lactating (4 days postparturition; L4) mice were used in this study. Pregnancies were timed by observing a vaginal plug (day 1 of pregnancy) after overnight residence with a male. The due date was calculated as 19 days after observation of the vaginal plug. Mice were housed under a 12-h light–12-h dark cycle and maintained on either breeder's chow (Teklad S-2335, no. 7004 mouse breeder diet; Harlan Teklad, Madison, Wis.) or a standard diet for postbreeding females (Teklad 22/5, no. 8640 rodent diet; Harlan Teklad, Madison, Wis.) and tap water ad libitum. All mice were anesthetized by intraperitoneal injection with avertin (125 to 250 mg/kg) and sacrificed by cervical dislocation. All procedures were approved by the Internal Animal Care and Use Committee of the University of Colorado.

**Adenovirus vectors.** Adenovirus vectors were grown in 293 cells, which are transformed by and express high levels of the adenovirus type 5 E1A and E1B proteins (4). A replication-defective adenovirus type 5 vector encoding enhanced, humanized, red-shifted green fluorescent protein under the control of the human cytomegalovirus major immediate-early promoter (Ad5GFP) was described previously (19).

**Virus growth.** Viruses were grown in 293 cells in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 10% bovine calf serum. For growth of high-titer stocks, 293 cells were infected and harvested by centrifugation at the time of maximal cytopathic effect, and the virus was released by three cycles of freezing and thawing. Cell debris was pelleted, the supernatant was saved, and the pellet was resuspended in phosphate-buffered saline (PBS), frozen and thawed, and pelleted. The supernatant was combined with the first supernatant. The pellet was resuspended in PBS and pelleted. The supernatant was combined with the prior supernatants. The supernatants were overlaid on a step gradient consisting of 1.25 and 1.4 g of CsCl per ml in PBS and centrifuged for 50 min at 36,000 rpm in an SW41 rotor (Beckman). The virus band was collected by side puncture, diluted with 1.35 g of CsCl per ml in PBS, and centrifuged for 3 h at 65,000 rpm in a VTi65 rotor (Beckman). The virus band was collected by side puncture, dialyzed for 1 h each against three changes of adenovirus storage buffer (10 mM Tris-HCl [pH 8.0], 135 mM NaCl, 1 mM $MgCl_2$, 50% [vol/vol] glycerol), and stored at −20°C until use. The concentration of virus particles was determined from the absorption at 260 nm, with 1 $A_{260}$ unit being equivalent to $10^{12}$ particles. Virus stocks were plaque titered on 293 cells.

**Adenovirus microinjection.** Ad5GFP microinjection was performed under avertin anesthesia at various stages of mammary gland development (Table 1). A stock of $2.7 \times 10^8$ PFU/ml was made in adenovirus storage buffer. Final doses ($2.7 \times 10^7$ PFU for fourth mammary glands; $2 \times 10^6$ PFU for third mammary glands) were made by diluting the $2.7 \times 10^8$ PFU/ml stock with sterile filtered Ringer's solution (138 mM NaCl, 8.1 mM $Na_2HPO_4$, 1.2 mM $K_2HPO_4$, 2.7 mM $KH_2PO_4$, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$). This dilution was made immediately before the microinjection to ensure the stability of the adenovirus. The solution was loaded into a 25-μl Wiretrol II disposable glass micropipette with a stainless steel plunger (no. 5-000-2050; Drummond Scientific Company, Broomall, Pa.). The end was drawn and fire-polished into a fine tip of 60 to 75 μm. By using a micromanipulator, the tip was gently inserted into the teat canal, and the solution was slowly ejected into the lumen of the either the third or fourth mammary gland as previously described (14). To evaluate the reliability of the injection technique, sterile filtered Ringer's solution was injected into contralateral control glands in some experiments.

TABLE 1. Experimental design

| Reproductive stage | | Total microinjection vol (μl) |
|---|---|---|
| Injection | End point[a] | |
| Nulliparous | 3 | 5 |
| P3 | P6 | 80 |
| P17 | L2 | 80 |
| P17 | L5 | 80 |
| P17 | L10 | 80 |
| P17 | L11 | 80 |
| P17 | L15 | 80 |
| P17 | L27 | 80 |
| L4 | L7 | 80 |

[a] Days after injection, pregnancy day, or lactation day.

**Determination of mammary epithelium permeability.** Since increased [$^{14}$C]sucrose permeability is one of the hallmarks of mastitis (9), we used this isotope to determine mammary epithelium permeability. On the day of sacrifice, 2 μCi of [$^{14}$C]sucrose (Amersham, Buckinghamshire, United Kingdom) were lyophilized and dissolved in sterile Ringer's solution. Then 5 μl (nulliparous glands), 40 μl (fourth mammary glands), or 20 μl (third mammary glands) of this solution was injected intraductally into the lumens of adenovirus-injected and contralateral control mammary glands of each mouse under avertin anesthesia. The 10-μl blood samples were taken from the tail vein 5 min after each injection, and the amount of $^{14}$C present was determined by liquid scintillation counting.

**Preparation of tissue for freezing and histology.** Injected experimental and contralateral control glands were excised and cut horizontally in half. One half of the gland was fixed in formalin, embedded in paraffin, and cut and stained with hematoxylin and eosin for histological purposes. The other half was cut into four to six smaller pieces and placed in aluminum foil molds filled with embedding medium (Tissue-Tek O.C.T. compound no. 4583; Sakura Finetek U.S.A., Inc, Torrance, Calif.) for frozen tissue specimens. The molds were flash frozen by immersion in an isopentane bath brought to its cooling point with liquid nitrogen.

**Frozen sectioning and immunohistochemistry.** Coverslips (Fisher Scientific no. 12-544-10) were treated with BD Cell-Tak and tissue adhesive (BD Biosciences no. 354240), rinsed, and stored overnight at 4°C. Then 12-μm sections were cut from the frozen molds with a cryostat at −32°C and collected onto the treated coverslips. The samples were placed at 37°C for 1 h and fixed in 2% paraformaldehyde (no. 00380; Polysciences, Inc., Warrington, Pa.) for 10 min.

After rinsing two to three times with PBS, the samples were treated with a blocking solution of 5% normal goat serum (#005-000-121; Jackson Immunoresearch, West Grove, Pa.) and 100 μg of saponin per ml (no. S4521; Sigma, St. Louis, Mo.). Samples were rinsed twice with PBS and incubated with the appropriate primary antibody for 1 h. A polyclonal antibody (7781) was made by using casein precipitated at pH 6.3 from mouse milk. Western blots showed specificity for mouse β-casein. Antibody against xanthine oxidase was generated against purified mouse xanthine oxidase in rabbit and purified on protein A-Sepharose (10). For nonantibody staining, samples were treated with wheat germ agglutinin conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) to outline the luminal surface of mammary epithelial cells, and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma D-9542) diluted in PBS was used to stain for nuclei.

Samples incubated with a primary antibody were rinsed five times for 5 min each with PBS and treated with both donkey anti-rabbit IgG conjugated to rhodamine (Molecular Probes; Eugene, Oreg.) and DAPI diluted in PBS. Both antibody-treated and non-antibody-treated samples were then rinsed six times for 5 min each in PBS. Then 60 μl of mounting medium (ProLong antifade kit, no. P-7481; Molecular Probes, Eugene, Oreg.) were placed on slides (Fisherbrand Superfrost, no. 12-550-15; Fisher Scientific, Pittsburgh, Pa.), and the coverslips were carefully lowered onto each slide. The slides were kept in the dark overnight and then placed at 4°C for storage.

**Determination of mastitis.** We developed a mastitis scoring system to examine the inflammatory response in Ad5GFP-transduced mammary epithelium. Three randomly chosen fields from hematoxylin and eosin-stained slides were assessed by bright-field microscopy at 40× magnification from various samples for the number of polymorphonuclear cells, mononuclear cell infiltration (scored 0, 1, and 2), and epithelial organization, again on a subjective scale (scored 0, 1, and 2), where 1 represents some mononuclear cell infiltration and epithelial disor-

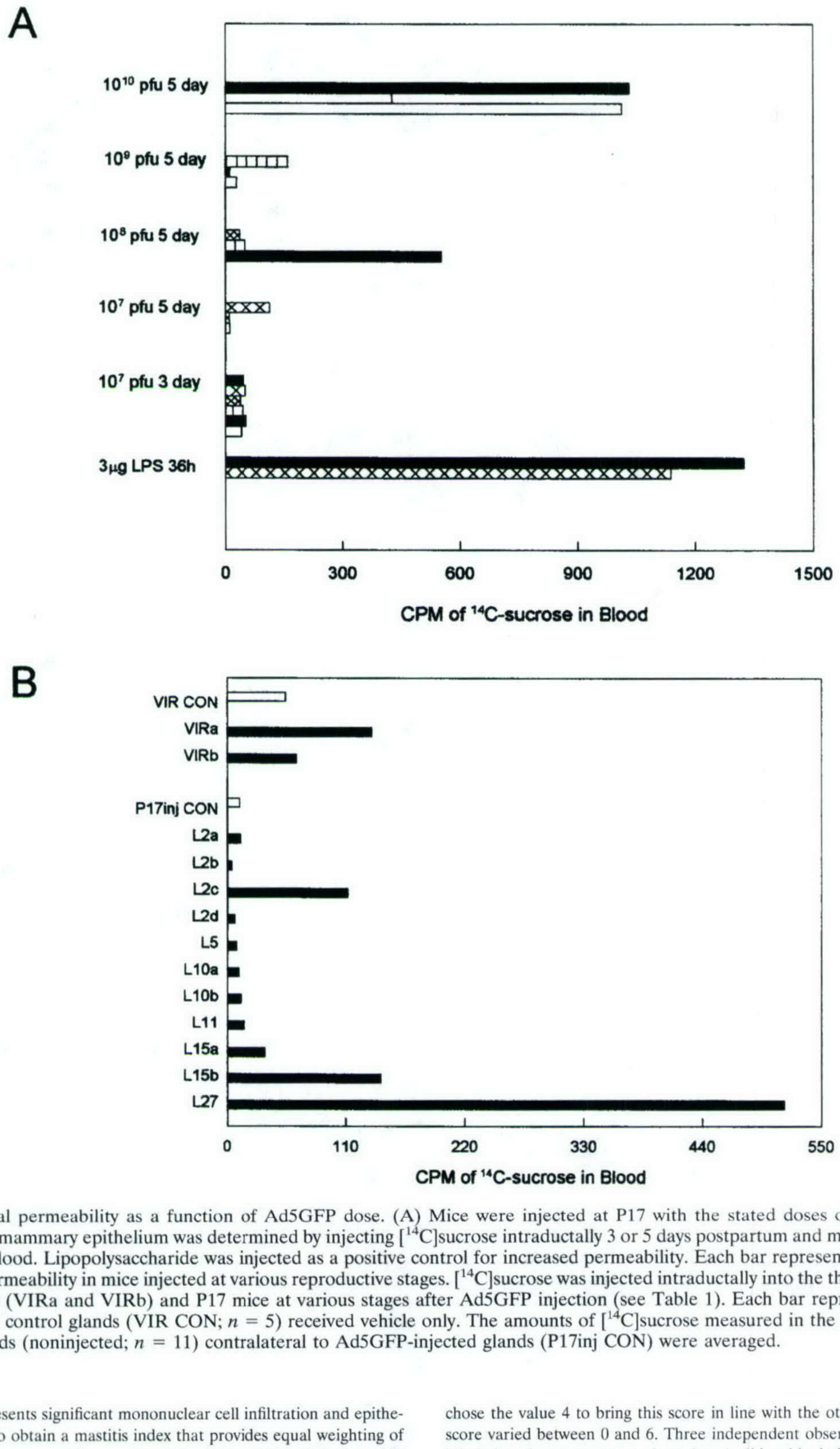



FIG. 1. Epithelial permeability as a function of Ad5GFP dose. (A) Mice were injected at P17 with the stated doses of Ad5GFP, and the permeability of the mammary epithelium was determined by injecting [$^{14}$C]sucrose intraductally 3 or 5 days postpartum and measuring the amount of $^{14}$C in 10 μl of blood. Lipopolysaccharide was injected as a positive control for increased permeability. Each bar represents a different mouse. (B) [$^{14}$C]sucrose permeability in mice injected at various reproductive stages. [$^{14}$C]sucrose was injected intraductally into the third mammary glands of nulliparous mice (VIRa and VIRb) and P17 mice at various stages after Ad5GFP injection (see Table 1). Each bar represents an individual mouse. Nulliparous control glands (VIR CON; $n = 5$) received vehicle only. The amounts of [$^{14}$C]sucrose measured in the blood after injection of P17 control glands (noninjected; $n = 11$) contralateral to Ad5GFP-injected glands (P17inj CON) were averaged.

ganization and 2 represents significant mononuclear cell infiltration and epithelial disorganization. To obtain a mastitis index that provides equal weighting of all three measurements, we used the equation MI = P/4 + MC + EO, where MI is the mastitis index, P is the average number of polymorphonuclear cells, MC is the mononuclear cell infiltrate, and EO is the average epithelial organization score. Because 9 was the maximal polymorphonuclear cell count per field, we chose the value 4 to bring this score in line with the others. Thus, the mastitis score varied between 0 and 6. Three independent observers, one of whom was blinded to the treatment, evaluated each slide with similar results.

**Microscopy and quantification of transduction.** For an initial assessment of Ad5GFP transduction, the glands were visualized under a Nikon dissecting microscope under fluorescent light. Digital images were captured by using Ax-

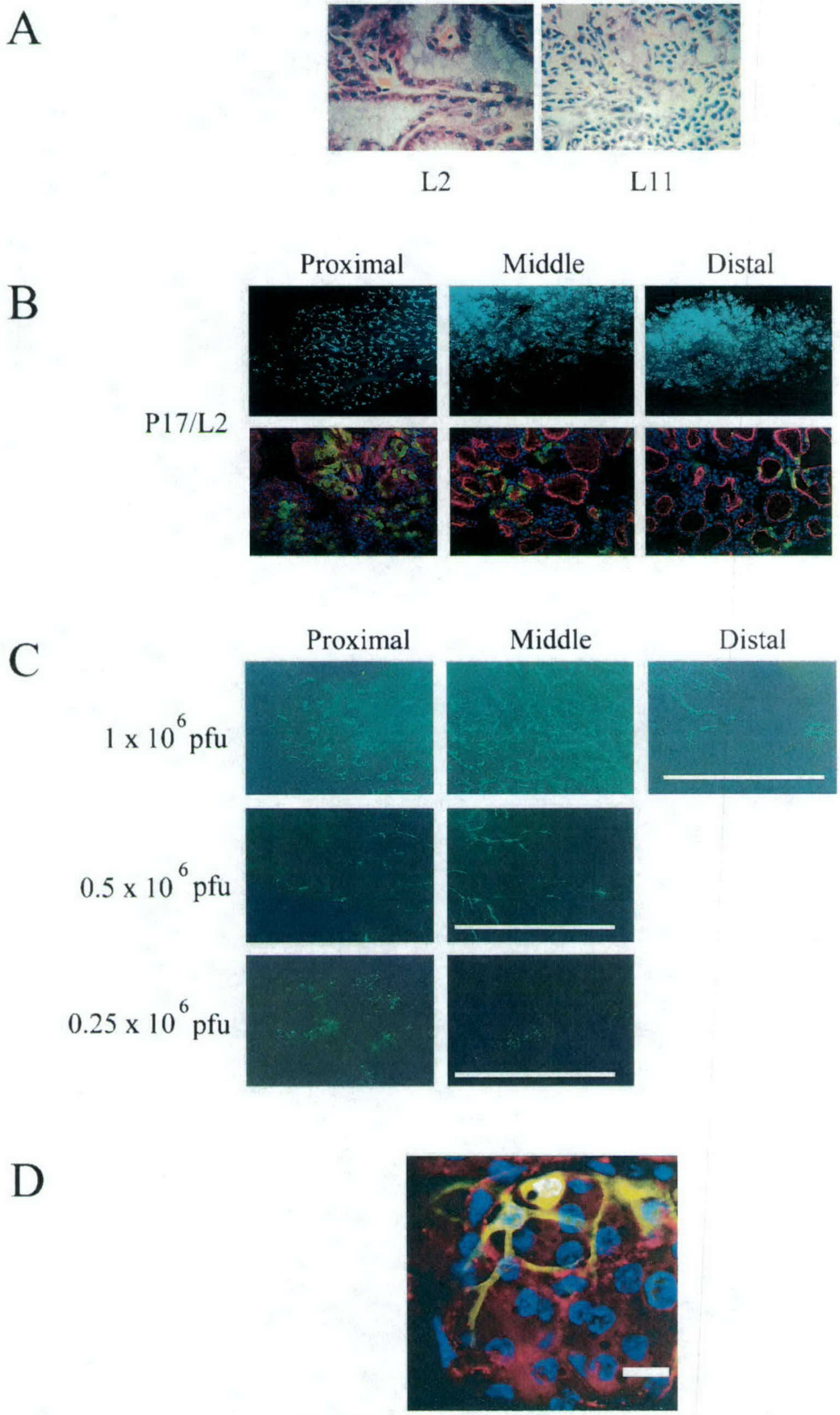



FIG. 2. Histological evidence of mastitis and extent of Ad5GFP transduction in nulliparous animals and animals injected during late pregnancy. (A) Hematoxylin and eosin-stained sections of third mammary glands from mice injected at day 17 of pregnancy (P17) and sacrificed on days 2 (L2) and 11 (L11) of lactation. (B) Initial assessment of transduction in whole glands in regions relative to the teat (proximal, middle, and distal) examined under fluorescent light (upper panel), and 12-μm sections examined under a confocal microscope (lower panel). (C) Whole glands from three individual nulliparous mice injected with different doses of Ad5GFP examined under fluorescent light. Bars, 5 mm. (D) Transduced myoepithelial cell. Bar, 20 μm.

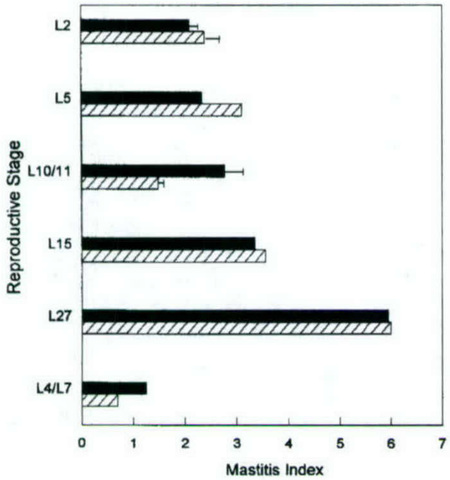



FIG. 3. Evaluation of mastitis. Data represent animals injected with Ad5GFP at P17 and sacrificed at various stages of mammary gland development (solid bars) and their respective controls (noninjected contralateral glands; hatched bars): L2 ($n = 3$), L5 ($n = 2$), L10/11 ($n = 3$), L15 ($n = 2$), L27 ($n = 2$), and L4/L7 ($n = 2$). Glands were assessed at 40× magnification by bright-field microscopy as described in Materials and Methods. See Materials and Methods for definition of the mastitis index.

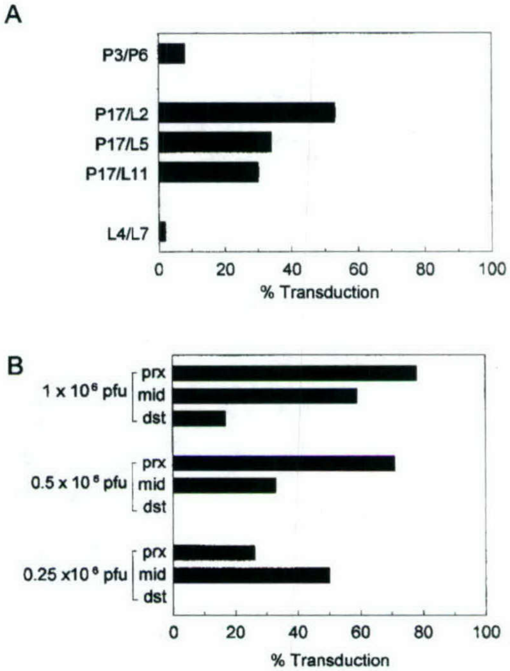


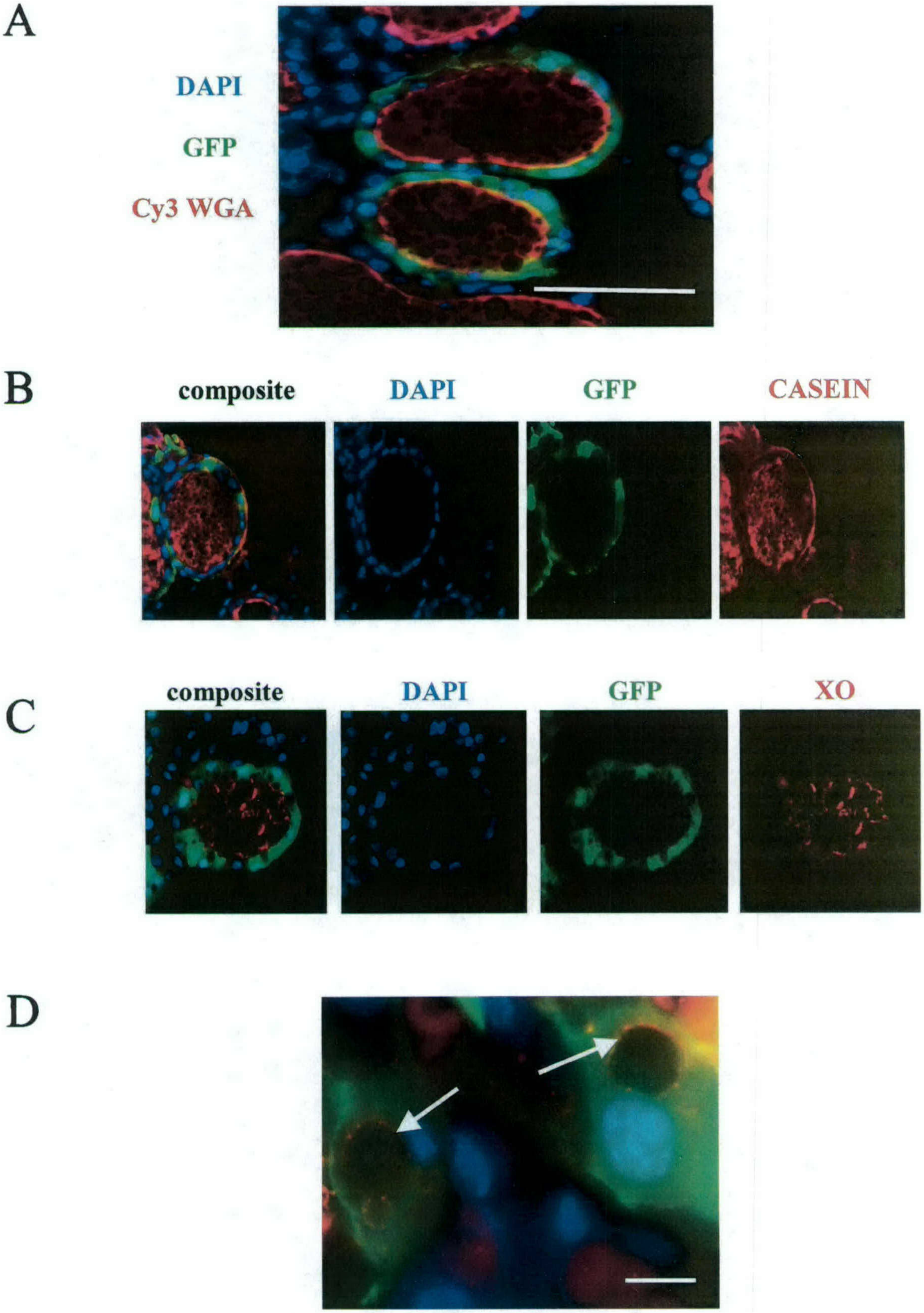

FIG. 4. Percent transduction in early pregnant, late pregnant, and nulliparous glands injected with AdGFP. (A) Percentage of epithelial structures (ducts and alveoli) showing transduction during mammary gland development. Each bar represents an average for three regions of the mammary gland (proximal, medial, and distal relative to the teat) at various stages of development: P3/P6 ($n = 2$), L2 ($n = 3$), L5 ($n = 2$), L10/11 ($n = 3$), and L4/L7 ($n = 2$). (B) Percent transduction of epithelial structures in nulliparous mice at different doses of Ad5GFP. Percentage of transduced epithelial structures was determined manually by counting the number of transduced and nontransduced epithelial structures in nulliparous mice injected with different doses of Ad5GFP: $1 \times 10^6$ PFU ($n = 2$), $0.5 \times 10^6$ PFU ($n = 2$), and $0.25 \times 10^6$ PFU ($n = 1$). Each bar represents the average for three sections in each segment (proximal [prx], medial [mid], and distal [dst] to the teat) of the gland.

ioVision software (Carl Zeiss, Inc., North America). A Nikon fluorescent confocal microscope was used to visualize immunohistochemical samples, and Slidebook software (Intelligent Imaging Innovations, Denver, Colo.) was used to capture the higher-power images. Percent transduction of mammary alveoli and/or epithelial structures was determined by manually counting transduced and nontransduced structures in three segments of the gland relative to the teat (proximal, medial, and distal). To determine the extent of transduction of epithelial cells, images were captured at identical magnifications and exposure times and quantified by using a masking program in Slidebook that was capable of determining either the proportion of transduced epithelium or the proportion of transduced nuclei by using DAPI-stained nuclei as a reference.

## RESULTS

**Adenovirus dosage determination.** To determine the maximal dose of adenovirus vector that could be injected without induction of inflammation, various amounts of Ad5GFP were injected intraductally into the fourth mammary glands of P17 mice. We assessed permeability by injecting [$^{14}$C]sucrose intraductally and measuring its level in the blood (15, 16). Because lipopolysaccharide induces massive mastitis (20), we injected it as a positive control. As the data in Fig. 1A show, high blood levels of sucrose were associated with injection of lipopolysaccharide. An adenovirus dose of $10^{10}$ PFU also led to high sucrose permeability (Fig. 1A) as well as histological signs of mastitis (data not shown). Sucrose permeability was relatively low with adenovirus doses of $10^8$ and $10^9$ PFU (Fig. 1A), with the exception of one sample. At a dose of $10^7$ PFU, epithelial permeability remained near baseline (Fig. 1A) and the tissue showed no signs of mastitis (data not shown). A dose between $10^7$ and $10^8$ PFU ($2.7 \times 10^7$ PFU) was determined to be safe for the fourth mammary gland of the mouse. For experiments in which we used the third gland, which is about one-fifth the size of the fourth gland, we injected $2 \times 10^6$ PFU.

**Duration of transduction.** In the next set of experiments, we used the third mammary gland to assess the amount of damage incurred by the mammary epithelium at various times after injection in a late pregnant mouse. In some cases, we evaluated the proximal, medial, and distal portions of the gland relative to the teat. As before, intraductal [$^{14}$C]sucrose injection was used to measure the permeability of the mammary epithelium (Fig. 1B), and mastitis was evaluated in hematoxylin and eosin-stained sections (Fig. 2A). Figure 2A shows an L2 mammary gland with no detectable mastitis. An island of massive mononuclear cell infiltration observed in a gland of a mouse sacrificed on lactation day 11 (L11), 12 days after adenovirus treatment, is also shown in the figure. A mastitis scoring system was designed to provide a semiquantitative measure of the extent of damage. This score, the mastitis index (measured as described in Materials and Methods), is shown for various times after injection in Fig. 3 and, in general, was not different from the index in the contralateral gland The L10 results were confirmed by three observers, but the [$^{14}$C]sucrose permeability data suggest that the degree of mastitis in the Ad5GFP-injected gland was small. Both the sucrose permeability data and the mastitis score show that an optimal dose of adenovirus produces little damage when injected into late pregnant mice


A
DAPI
GFP
Cy3 WGA
B
composite
DAPI
GFP
CASEIN
C
composite
DAPI
GFP
XO
D

and left up to 5 days or longer into lactation (Fig. 1B and 3). By L27, the gland becomes disorganized due to involutional remodeling (17).

We also examined Ad5GFP transduction in mammary glands from nulliparous mice. We found that volumes larger than 5 μl burst the small ductal system in these glands (data not shown), so all adenovirus injections were restricted to this volume. As a control, 5 μl of sterile-filtered Ringer's solution was injected into the contralateral gland. Three independent observers, one blinded, concluded that there were no gross differences between the control and injected glands and that mastitis appeared relatively minimal (data not shown). The extent of transduction in early pregnant and lactating glands was very low (see below), so mastitis was not formally evaluated in these conditions.

**Extent of adenovirus transduction.** We viewed transduced mammary glands at low magnification to obtain an initial assessment of the amount of Ad5GFP transduction (Fig. 2B, upper panel, and 2C). Although transduction appeared to be concentrated in areas proximal to the teat or in the middle of the gland in most nulliparous and pregnant animals, some glands did exhibit widespread transduction, as shown in Fig. 2B and 2C (upper panels). We assessed mammary sections from pregnant and lactating mice by using a 20× confocal view of 12-μm-thick frozen sections, first counting the proportion of ducts and alveoli that showed some transduction (Fig. 2B, lower panel). This analysis showed that many but not all alveolar structures were transduced. Less than 10% of the ductal and alveolar structures of glands from early pregnant mice showed some transduction, while approximately 30 to 50% of the structures were transduced when the virus was injected in glands from late pregnant mice (Fig. 4A). Very low transduction was achieved in glands from Ad5GFP-injected lactating mice (Fig. 4A).

To further investigate the extent of alveolar transduction, we used the masking function of Slidebook (see Materials and Methods). By using glands injected at P17 and sacrificed on day 2 of lactation, we were able to delineate specific cellular areas of transduction and calculate the proportion of total epithelium transduced in each alveolar structure. With this approach, we found that the percentage of transduced epithelium was highly variable between two different mice (data not shown). For example, approximately 25% of the total epithelium was transduced at this stage of mammary gland development in areas proximal to the teat in one animal, and only 7% of the total epithelium was transduced in the same area in another mouse. Areas medial and distal to the teat had lower percentages of total transduced epithelium that also varied greatly between animals (data not shown).

To get an approximate measure of the efficiency of transduction, an estimation of the number of epithelial cells in the third mammary gland was made and compared with the frequency of transduction. By using the DNA content of the gland as a starting point, it was estimated that there were approximately $3 \times 10^7$ epithelial cells. Approximately 20%, or $6 \times 10^6$, of these cells were transduced in the most highly transduced gland when $2 \times 10^6$ PFU of Ad5GFP were injected. The ratio of transducing units to PFU was near 5:1, so approximately $10^7$ transducing units were injected. This analysis suggests that greater than half of the injected vector transduced epithelial cells, supporting the idea that the process is relatively efficient during the late stage of pregnancy in the mammary gland.

The mammary gland consists of an inner layer of ductal cells and alveoli and an outer contractile monolayer of myoepithelial cells closely attached to the basement membrane. The myoepithelial cells extend laterally along ducts and form a basket-like sheath around both ducts and alveoli (13). In response to oxytocin binding to specific receptors, myoepithelial cells contract and expel milk from the alveoli into the ducts and eventually out of the gland. The presence of GFP in myoepithelial cells in glands transduced in late pregnancy (Fig. 2D) suggests that viral particles were able to traverse the paracellular compartments of the mammary gland or that some myoepithelial cells were exposed at the luminal surface. The presence of GFP in myoepithelial cells also raises the possibility that the basolateral surface of the mammary epithelium is accessible during late pregnancy, when the paracellular spaces are open to large molecules, allowing adenovirus access to its receptor (1, 23, 27).

Epithelial structures of nulliparous mice can also be transduced by Ad5GFP (Fig. 2C and 4B). Portions of the gland proximal to the teat were highly transduced after injection with relatively high doses of virus, but the extent of transduction was greatly reduced with decreasing doses (Fig. 2C and 4B). It is possible that adenovirus transduction can be used experimentally in nulliparous animals with small volumes and careful attention to optimizing the dose.

**Functional competence of transduced cells.** The data presented to this point show that mammary epithelial cells can be transduced with Ad5GFP during pregnancy and can be maintained well into lactation without inflammation. However, in order to utilize adenovirus microinjection as an effective method of changing gene expression, transduced cells must retain functional integrity. Two distinct pathways of cellular milk synthesis, milk protein secretion and milk fat secretion, can be assessed morphologically. Transduced alveoli displayed normal morphology, produced and secreted milk fat globules, and were laden with milk, which was stained red in the lumen of the alveoli in Fig. 5A. Casein, a milk protein, was also produced and was detected in the lumen of transduced alveoli (Fig. 5B). Xanthine oxidase has been shown to redistribute from the cytoplasm to the surface of emerging milk fat globules during pregnancy at the onset of lactation, and this redistribution is thought to be essential for milk fat globule release (J.

FIG. 5. Maintenance of function in mammary glands transduced with Ad5GFP. (A) Two nearly completely transduced (green) alveoli showing milk oligosaccharides stained with rhodamine-wheat germ agglutinin (red), nuclei stained with DAPI (blue), and surrounding milk lipid droplets. These alveoli appear morphologically normal. Bar, 100 μm. (B) A 20× view of lumens of transduced (green) alveoli stained with anticasein antibody (red); the nuclei were stained with DAPI (blue). (C) A 20× and (D) a 100× view of fat droplets in transduced (green) alveoli rimmed with xanthine oxidase (XO) as shown by stain (red) with an anti-xanthine oxidase antibody; the nuclei are stained with DAPI (blue). Arrows indicate milk fat droplets rimmed with xanthine oxidase in panel D. Bar, 20 μm.

McManaman, personal communication). This localization of xanthine oxidase was maintained in transduced cells (Fig. 5C and 5D). Thus, the transduced cells appeared to be fully functional.

## DISCUSSION

Adenovirus vectors have been used to transduce a variety of organs in a number of animal species. Mice have been especially popular as models to study the effects of adenovirus vectors. Intraductal injection of adenovirus vectors provides a versatile method of altering gene expression in both the luminal and, to some extent, the myoepithelial cells of mouse mammary glands. In no case did we find transduction in the interstitial spaces of the mammary glands that we studied. It is possible to transduce nulliparous glands (Fig. 2C and 4B), even though these glands are immature compared to late pregnant glands and are particularly smaller in size. Although adenovirus can be used to transduce the epithelium at any stage of mammary gland development except lactation, the technique has proven to be most effective during the switch from pregnancy to lactation (Fig. 2B, 3, 4A, and 5A). Little mastitis was observed up to a week to 10 days after transduction with an optimal dose of virus (Fig. 1 and 3). The extent of transduction was highly variable within regions of the gland relative to the teat and may not generate enough material for biochemical analysis of transduced cells. Nevertheless, morphological studies may still be possible with neighboring nontransduced cells as controls.

We had less success with adenovirus transduction at other stages of mammary gland development. Transduction during early pregnancy was relatively low (Fig. 4A), possibly because the epithelium is turning over rapidly at this developmental stage (2). Transduction during lactation (Fig. 4A) was rarely successful. Our laboratory has previously shown that tight junction permeability is very low during lactation (16), preventing solute passage through the paracellular pathway to the basal surface of the gland, where the adenovirus receptor may be localized (23, 27) There is also evidence that the coxsackie and adenovirus receptor is a component of the tight junction complex and that its localization within this complex impedes viral transduction (3).

Taking these factors into consideration, it is possible that the change in tight junction permeability that accompanies secretory activation is responsible for the low efficacy of adenovirus transduction during lactation. Also, during lactation, the high concentration of milk proteins may adsorb the viral particles. The density of adenovirus receptors may also differ at different stages of mammary gland development with changes in endocrine state, and these differences may influence the efficacy of adenovirus transduction (7). Thorough investigations with such techniques as in situ hybridization and adenovirus receptor density studies in mouse mammary glands at various stages of development are needed to properly address these issues. Nevertheless, adenovirus vectors appear to be effective in mediating the transduction of functional genes into mouse mammary epithelial cells, particularly ain late pregnancy, when the tight junctions between epithelial cells are open to the passage of large molecules (15).

Our results also demonstrate that transduction with Ad5GFP does not disrupt normal mammary epithelial cell morphology and function (Fig. 5). Adenovirus transduction did not disrupt either of two major and distinct synthetic pathways for cellular milk secretion, milk protein secretion and milk fat globule formation. These data clearly show that adenovirus transduction can be used to alter gene expression and to study luminal cell function in the mouse mammary epithelium.

Adenovirus vectors used in sufficient amounts to efficiently transduce target tissues generally induce a strong inflammatory response that typically reaches a high level within 4 to 7 days of vector introduction in a variety of immunocompetent mouse strains. Inflammation occurs within targeted organs transduced via the bloodstream, such as the liver (25), as well as when the mucosal surface of an organ is targeted, such as in the lung (26). The inflammatory response both limits the duration of expression and leads to an immune response that limits successful reuse of the vector. The relatively rapid induction of inflammation has limited the usefulness of adenovirus vectors for studies of altered gene expression in most tissues. Thus, the findings presented here are somewhat surprising.

It is not clear why the induction of inflammation is delayed when adenovirus vectors are used in sufficient amounts to transduce a significant fraction of the mammary epithelium. It is possible that this delay is due to the use of outbred CD-1 mice. Adult CD-1 mice have been successfully transduced for extended periods of time with adenovirus vectors (22), and neonatal CD-1 mice did not exhibit inflammation after injection with an adenovirus vector (21), raising the possibility that CD-1 mice may exhibit reduced inflammatory and immune responses to adenovirus vectors relative to other mouse strains. Alternatively, it may be that pregnancy reduced the immunocompetence and inflammatory response of the mice. Regardless, the delayed onset of inflammation means that adenovirus vectors can be effectively used as a tool for altering gene expression in the mammary gland of CD-1 mice.

The usefulness of first-generation adenovirus vectors in transduction of the mammary epithelium of late pregnancy means that it is not necessary to undertake the substantial efforts required for the use of helper-dependent, or "gutless," vectors. However, it remains a possibility that the use of second-generation vectors made replication incompetent through deletion of a viral gene essential for viral DNA replication, such as the terminal protein gene (11), will lead to a reduction in the low level of inflammation or extend the time prior to the appearance of inflammation, extending their usefulness. Since such vectors are relatively easy to construct and grow, it may be worthwhile to test their effectiveness in transducing the mammary epithelium.

Intraductal microinjection of adenovirus vectors should aid in studies of a variety of genes of interest in the mammary epithelium. Since adenovirus transduction appears to be confined to the mammary epithelium, these methods provide a technique to target genes of interest to this tissue compartment. Potentially, these techniques could be used for drug, hormone, or protein delivery to milk on a short-term basis. Currently, our laboratory is preparing to use the methods described here to examine the regulation of milk synthesis and secretion in the mouse mammary epithelium. Recombinant adenovirus constructs could potentially target genes responsible for certain types of breast cancers. The procedures de-

scribed here provide a means of studying the efficacy of such vectors.

## ACKNOWLEDGMENTS

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# Claudin-7 is not just a tight junction protein: Expression and localization in the normal murine mammary gland and murine mammary tumors.

[1]Brigitte Blackman, [1]Tanya Russell, [1]Steven K. Nordeen, [2]Daniel Medina, and [1]Margaret C. Neville

[1]University of Colorado Health Sciences Center, Denver, Colorado 80262

[2]Baylor College of Medicine, Houston, Texas

*Correspondance should be addressed to MCN at:*

Department of Physiology and Biophysics

University of Colorado Health Sciences Center

4200 E. Ninth Ave.

Box C240, Room 3802

Denver

CO 80262

Phone: 303 315 8230

Fax: 303 315 8110

E-mail: peggy.neville@UCHSC.edu

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**Summary**

Claudins are normally associated with the tight junctions of epithelial cells where they confer a variety of permeability properties to the transepithelial barrier. We examined the developmental expression and localization of claudin 7 in the murine mammary epithelium. The molecule is constitutively expressed in the mammary epithelium at all levels of development and the ratio of its mRNA expression to that of keratin 19 was nearly constant. By immunohistochemistry at all developmental stages claudin 7 was located in the basolateral part of the cell where it was found in discreet cytoplasmic spots presumably representing vesicles and at the cell border. At high magnification no colocalization with the tight junction protein, ZO1 could be observed. We confirmed the basolateral localization of claudin 7 in the airway epithelium and some types of renal tubules where cytoplasmic vesicles were also observed. That claudin 7 can associate with tight junctions was shown by its colocalization with the tight junction scaffolding protein, ZO1 in EPH4 cells, a normal murine mammary cell line, and in the epididymus. It was also localized in the cytoplasm of MMTV-neu murine mammary tumors as well as in the transplantable murine tumor cell lines, TM4, TM10 and TM40A, where its ratio to cytokeratin was higher than in the normal mammary epithelium. Our observations suggest that claudin 7 may function in mammary epithelial cells to regulate vesicle trafficking to the basolateral membrane or stabilization of cytoplasmic vesicles.

## Introduction

The claudins comprise a large family of tetraspanin membrane proteins thought to be the major barrier forming proteins of tight junctions, the cell-cell contacts at the apical border of epithelial cells that control the paracellular movement of solutes. These proteins are highly conserved with four transmembrane domains and two hydrophobic extracellular loops; the latter are thought to mediate cell-cell adhesion (Kubota et al., 1999) and to confer specific paracellular permability properties on cell monolayers (Colegio et al., 2002, 2003). Claudin 7 shares the general structural characteristics of the family, differing primarily in its N-terminal cytoplasmic tail (Morita et al., 1999). The molecule has been shown to be associated with epithelial cells in the human breast (Kominsky et al., 2003) and its loss is associated with some breast and head and neck malignancies (Al Moustafa et al., 2002; Kominsky et al., 2003). It has been shown to be expressed in parts of the renal tubule (Li et al., 2004) and the airway epithelium (Coyne et al., 2003), where it is localized to the basolateral aspects of the cells. Here we show that claudin 7 is constitutively present in the epithelium of the murine mammary gland, again localized, not to tight junctions, but to punctuate structures at or near the basolateral surfaces of the cells. It was present at all cell borders of several murine mammary tumors. However, the protein can localize to tight junctions, as shown by its colocalization with ZO1 in cultured mammary epithelial cells and epidydimus, suggesting a possible dual function depending on tissue type.

## Materials and Methods

*Animals and Tissue preparation.*

CD-1 mice, purchased from Charles River Breeding laboratory, Wilmington, were maintained in the USDA approved Animal Resource Center of the University of Colorado Health Sciences Center. All procedures were approved by the Institutional Animal Care and Use Committee. The fourth mammary glands of virgin female mice at 3, 6, and 12 weeks; female mice during early gestation (5–7 days), midgestation (12 days) and late gestation (18 days); at day 2 and 10 of lactation and day 21 and 29 of involution were collected after euthanasia involving a lethal dose of pentobarbitol. Liver, lung, and kidneys were obtained from virgin female mice and epididymus from male mice. The day vaginal plugs were observed was counted as day one of pregnancy. Mice overexpressing the *Her2/neu* oncogene (Guy et al., 1992) were obtained from Jackson laboratories and mammary tumors were dissected and frozen when they reached about 0.5 cm in diameter. Transplantation tumor models; TM4, TM10L and TM40 were obtained from Dr D. Medina (Baylor College, Houston, TX; Medina et al., 1993). Tissues were flash frozen in liquid nitrogen in TRI-ZOL reagent (GIBCO BRL, Life Technologies) for total RNA purification. For protein extractions, the tissue was washed twice in 1ml PBS and then placed in 3 volumes of 1% NP-40 buffer (25mM Hepes/NaOH, pH7.4; 150 mM NaCl, 4mM EDTA; 1% NP-40) containing protease inhibitors (10μg/μl leupeptin, 10μg/μl aprotinin, 10μM pepstatin A, 1mM PMSF, in DMSO). For *in situ* hybridizations and immunohistochemistry of frozen tissue, tissues were placed in Tissue-Tek O.C.T. 4583 Compound (Sakura, Torrance, CA) and flash frozen in isopentane and liquid nitrogen. All samples were stored at –70°Cuntil ready for use.

*Characterization of an antibody to claudin 7.*

A rabbit antibody was custom made against the C-terminal peptide of murine claudin 7, APRSYPKSNSSKEYV, by Zymed, Laboratories, San Francisco, CA. This affinity purified antibody bound only a 23 Kd peptide by Western blot (Figure 2A), did not cross-react with claudin 1, its closest congener, stained only epithelium where its mRNA has been demonstrated and stain was blocked by preincubation of the antibody with the peptide. Cos cells transfected with full length claudin 7 stained with this antibody but not an antibody to its closest congener, claudin 1.

*Real time PCR protocol.*

For real time RT-PCR RNA was purifed using the Quiagen system; 1 ug DNAase treated RNA was used for each reaction. Triplicate tissue samples were assayed in duplicate using the ABI Prism 7700 sequence detection system with lung as the positive control. Primer sequences: Murine Claudin 7, Forward CGAAGAAGGCCCGAATAGCT (338 – 337); reverse GCTACCAAGGCAGCAAGACC (407 – 388); probe GCCACAATGAAAACAATGCCTCCAGTCA (359-386). Murine cytokeratin: forward, TTTAAGACCATCGAGGAC, reverse TCATACTGACTTCTCATCTCAC.

*Immunohistochemistry and digital confocal microscopy.*

Rat anti-mouse ZO1 antibody was obtained from Chemicon International, Inc., Temecula, CA. Tissue sections, cut at 10 µm, from frozen blocks on a Damon/IEC division minotome set at -18 to -20°C. Sections were collected onto Cell-Tak coated coverslips and were further vapor-fixed with paraformaldehyde for 15 minutes. Sections were never allowed to dry. PBS was carefully added so as not to disrupt the sections. Tissue was permeabilized with 1% Triton X100 for fifteen minutes, rinsed well with PBS

and blocked with sterifiltered, 10% normal donkey serum for 20 minutes. All antibody solutions were microfuged for 20 minutes prior to use. The claudin antibody was diluted 1:1000. Primary incubations were for an hour at room temperature, followed by extensive washes in PBS, generally six times five minutes each. Secondary antibodies were diluted according to manufacturer's instructions, in PBS alone. The host species of all secondary antibodies was donkey and all secondary antibodies were cross-adsorbed against mouse serum proteins. Antibodies used were conjugated to fluorescein, CY3 or CY5. Secondary antibodies were combined with 0.6 μg/ml DAPI, and incubated on the tissue for an hour. Coverslips were rinsed briefly and allowed to soak overnight in PBS.

Images were collected using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO) on a Nikon Diaphot TMD microscope equipped for fluorescence with a Xenon lamp and filter wheels (Sutter Instruments, Novato, CA), fluorescent filters (Chroma, Brattleboro, VT), cooled CCD camera (Cooke, Tonawanda, NY) and stepper motor (Intelligent Imaging Innovations, Inc., Denver, CO). Multi-fluor images were merged, deconvolved, and renormalized using SlideBook software.

## Results

*Developmental expression of Claudin 7 mRNA in the mammary gland.*

Fig. 1A shows claudin 7 gene expression as a function of developmental stage in the mouse mammary gland from the virgin animal (NPNL) through pregnancy (P5, P12 and P18), lactation (L2, L10, L19), and involution (L22, L29). Gene expression increased more than 1000 fold between the virgin and early lactating gland, leveling out through lactation and decreasing at late involution (L29) with the loss of epithelial cells. To determine whether expression of the claudin 7 was a function of developmental stage or epithelial cell number, so we also examined the expression of keratin 19, found only in luminal epithelial cells (Gudjonsson et al., 2002). As can be seen, claudin-7 expression parallels that of keratin 19, and the ratio of claudin-7 to keratin 19 (dotted line, Fig. 1A) is relatively constant through pregnancy and early lactation, increasing only during later lactation when the expression of keratin 19 mRNA decreases significantly. Similar results were obtained from microarray analysis (data not shown).

At late involution, day 29, claudin 7 expression was lower than that of keratin 19, possibly having to do with the types and characteristics of epithelial cells present during late glandular remodeling. In situ hybridization with $^{35}S$ labeled RNA probes to claudin 7 showed that the mRNA was localized to the epithelium in virgin, pregnant and lactating animals (Fig. 1B). We conclude that, in the normal mammary gland, claudin 7 is confined to the epithelial compartment and is expressed at approximately constant levels through development. The very large changes in tissue expression levels during pregnancy most likely reflect an increase in epithelial cell number as the mammary epithelium expands from a system of sparse ducts in the virgin gland, to a dense mass of lactating cells in a gland where the adipose tissue is largely obliterated.

*Immunolocialization of claudin 7 in the mammary gland.*

We made and characterized an affinity purified antibody to the cytoplasmic tail of claudin 7 (see Methods) that proved satisfactory for both Western blots and immunohistochemistry on both frozen and paraffin sections (Fig. 2). Given the well-known association of claudins with tight junctions, we were surprised to find that claudin stained the basolateral region of mammary cells in all stages of development (Fig. 2). A ductal structure surrounded by adipose stroma is shown from a virgin animal; alveoli from the glands of pregnant and lactating animals are also shown (Fig. 2B). Staining was blocked when the antibody was preabsorbed with the claudin-7 peptide against which it was made (Fig. 2B, lower right). Consistent with the in situ analysis, stain was observed only in luminal epithelial cells. Although there appears to be some colocalization of claudin 7 with stain for the tight junction scaffolding protein ZO1 in the lower power images of Fig. 2B, at higher magnification (Fig. 2C) claudin 7 was entirely localized to the basal and lateral cytoplasmic regions where it often appeared punctate in nature, particularly when it was not closely apposed to the cell border. Stain was excluded from nuclei (blue) and tight junctions (green) as shown by the lack of overlap between the green and red stain in Fig. 2C. It seems likely that there is basolateral membrane staining in this tissue, but the basal and lateral membranes of the mammary epithelial cells are deeply infolded and membrane localization cannot be assessed at the magnifications possible with the light microscope.

*Claudin 7 localization in other cell types*

Sukumar and colleagues (Kominsky et al., 2003) reported that claudin-7 was localized to tight junctions of the human mammary carcinoma cell line, MCF-7, using an antibody directed toward the cytoplasmic tail of the human protein which differs by one amino

acid from the mouse protein. We therefore examined claudin-7 staining in the normal mouse mammary cell line, EPH4 (Matsuda et al, 2004; Reichmann et al, 1992) comparing its distribution with that of ZO1 (Fig. 3A). Claudin 7 distinctly colocalizes with ZO-1 with a slightly less compact distribution. A scattering of cytoplasmic vesicles can also be seen. This finding clearly indicates that claudin 7 is capable of localizing to tight junctions, a conclusion supported by claudin 7 distribution in the epidydimus (Fig. 3B) where claudin-7 colocalized with ZO-1 at the apical borders of some cells, but not others. Punctate cytoplasmic stain for claudin-7 can also be observed in some cells.

Claudin-7 mRNA was reported in lung and kidney, with liver being negative (Morita et al., 1999). We therefore examined these tissues as well, finding that claudin 7 was present in cells lining bronchiolar structures of the lung where its distribution was mainly cytoplasmic as observed previously (Coyne et al., 2003; Fig. 3C). However, careful examination of a black and white image at the highest magnification in the luminal cells of this structure shows some claudin-7 to be colocalized with ZO-1, suggesting that it may also be associated with tight junctions in these cells. There was no stain in the pulmonary alveoli. In the renal cortex we found claudin 7 localized only to certain segments, identified as connecting tubules and cortical collecting ducts by Li et al (Li et al., 2004), where stain was distinctly basolateral (Fig. 3D). Since our antibody is different from the one used by Li et al., our finding confirms this localization. At high magnification the stain is punctate as in the mammary and airway epithelia. No specific claudin 7 stain was observed in the liver (Fig. 3E). These findings indicate that claudin 7 is capable of localizing to tight junctions as in cultured mammary epithelial cells and epidydimus, but in mammary gland, airway and kidney it is mostly or entirely confined to punctate cytoplasmic structures, often near the basolateral surfaces of the cells and

possibly associated with the basolateral membranes. In no tissue was the protein observed in nuclei.

*Claudin 7 localization in mammary tumors.*

We examined four types of mammary tumors: tumors arising in the transgenic mouse expressing the Erb2 receptor under the control of the mammary tumor virus promoter (Guy et al., 1992) and three transplantable tumors obtained from the laboratory of Daniel Medina (Medina et al., 1993). All expressed claudin 7 mRNA at levels no more than 2-fold higher than the lactating mammary gland when normalized to ribosomal RNA (Fig. 4A). Although tumors themselves are more closely related to the pregnant gland, we chose the lactating gland for comparison because, like the tumors, it is composed largely of epithelial cells. Interestingly, the ratio of claudin 7 to keratin was nearly 10-fold lower in the tumors than in the lactating gland, reflecting lower expression of keratin 19 in the tumors possibly reflecting a loss of differentiation. In all murine tumors examined here claudin 7 was localized to the perimembrane region as illustrated by a section through the TM4 tumor (Fig. 4B). None of these tumors demonstrated ZO1 staining indicating that they lack tight junctions, so that the claudin 7 observed here is likely associated with membrane vesicles and possibly basolateral membranes as in the normal cells of the mammary gland. Sukumar and her colleagues (Kominsky et al., 2003) observed a loss of claudin 7 expression in some human tumors, particularly lobular tumors. However, this finding was not true of the mouse mammary tumors examined.

**Discussion**

We find claudin 7 to be a constitutive component of luminal mammary epithelial cells, where it is localized to the basolateral regions of the cell. The finding that the ratio of its mRNA to that of keratin 19 is relatively constant throughout the developmental cycle,

suggests that the molecule may be an alternative marker to keratin for the proportion of epithelial cells in the mammary gland. It is not clear whether the more than 1000-fold increase in expression between the virgin gland and early lactation indicates that the number of epithelial cells increases in this proportion, since part of the increase could be a function of an increase in cell size as the cells differentiate. The rapid increase between the virgin gland and pregnancy day 5 is consistent with studies showing a peak of [$^3$H]-thymidine incorporation between days 2 and 5 of pregnancy when approximately 25% of the cells were labeled. (Borst and Mahoney, 1982; Traurig, 1967). In both studies labeling decreased after pregnancy day 5 but remained around 10% almost to parturition, consistent with the continued increase in both claudin 8 and keratin mRNA up to P18.

Our findings are consistent with images of claudin staining in the human mammary gland where a diffuse diaminobenzidene (DAB) stain from alkaline phosphatase localization was present throughout the cytoplasm (Kominsky et al., 2003). However, in that study no attempt was made to localize claudin-7 stain with tight junction components. Basolateral localization of other claudins has been observed. Claudin 1 was similarly localized in the epidydimus (Gregory et al., 2001), intestine (Walsh et al., 2001) and cornea (Ban et al., 2003), and we have observed claudin 1 both associated with tight junctions and basolaterally distributed in the luminal epithelium of the mouse mammary gland (unpublished data). Rahner et al. (2001) observed claudins 3, 4 and 5 to be laterally distributed in various portions of the gastrointestinal tract. The finding that claudin 7 is exclusively located in non-tight junction regions of mammary and renal epithelial cells (Li et al., 2004), suggests that claudins may have functions other than regulation of tight junction permeability. Our images appear to be the first to show claudin 7 stain at sufficiently high resolution to show punctate cytoplasmic stain in

mammary, airway and renal epithelial cells. Even at this resolution, obtained with digital confocal imaging with a resolution of about 200 nm, it is not possible to discern with certainty whether claudin 7 is inserted into the convoluted basolateral membranes of these cell types. If so, it is possible that the cytoplasmic spots represent vesicles on route to and from to these membranes, where claudin 7 may interact with components of the extracellular matrix. As a precedent, claudin 11, an oligodendrocyte protein has been shown to interact with $\alpha 1$-integrin and to regulate proliferation and migration of oligodendrocytes in culture (Tiwari-Woodruff et al., 2001). Other possibilities are that vesicular claudins could regulate tight junction permeability by sequestering tight junction regulatory molecules away from tight junctions, or they could be involved in the stabilization of specialized vesicle compartments within the cytoplasm. Matsuda and colleagues, using time lapse photography show that claudin containing cytoplasmic vesicles can originate from the tight junctions as the epithelial layer remodels (Matsuda et al, 2004). However, since claudin 7 is never observed associated with tight junctions in mammary epithelial cells, it seems unlikely that claudin 7 containing vesicles originate from the junctional complex in this tissue. Unfortunately, our mammary epithelial cell model, EPH4 cells, does not possess a heavy complement of cytoplasmic vesicles, so that live cell imaging studies will need to be carried out in the animal, to determine the origin and disposition of claudin 7 containing vesicles. It is possible that the function of the vesicles could be better assessed from analysis of the composition of the claudin 7 containing cytoplasmic vesicles.

Claudin 7 expression was inversely correlated with histological grade in a large series of breast tumors (Kominsky et al., 2003). These same authors found, similar to our observations with EPH4 cells, that claudin 7 colocalized with ZO1 in MCF7 breast

cancer cells and could also be observed in cytoplasmic spots. It was present in luminal cells of the human breast, where the DAB staining appears to be localized to basolateral membranes, although in the absence of an apical marker like ZO1, it is difficult to draw a firm conclusion. An image of ductal carcinoma in situ (DCIS) in that paper shows a distribution of stain remarkably similar to that of the TM10 tumor shown in Figure 4B. This tumor line has been shown to have a ductual morphology (Medina et al., 1993). Thus we conclude that low grade breast carcinomas show a cellular distribution of stain similar to that observed in the murine tumors. Interestingly, the murine tumors showed claudin 7 expression at the mRNA level equal to or higher than that of the lactating mammary gland, where the largest proportion of the cells are the luminal epithelial cells that give rise to mammary tumors. TM4, the most tumorigenic of these lines, had the highest claudin 7 expression, although the level was quite variable and not significantly different from the other lines examined. Interestingly TM4 and the MMTV-neu tumor had ratios of claudin 7 to cytokeratin more than 8 fold less than the slower growing TM10 line. All of the tumors had claudin 7/cytokeratin ratios significantly higher than the pregnant or lactating mammary gland. This finding suggests that loss of the cytoplasmic architectural stability conferred by keratin may be an early event in tumorigenesis. Together with the data from the Sukumar laboratory, we might speculate that loss of claudin 7 as occurs in high grade tumors, alters cell matrix interactions allowing a greater degree of cell mobility and contributing to metastasis

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**Figures and Legends.**

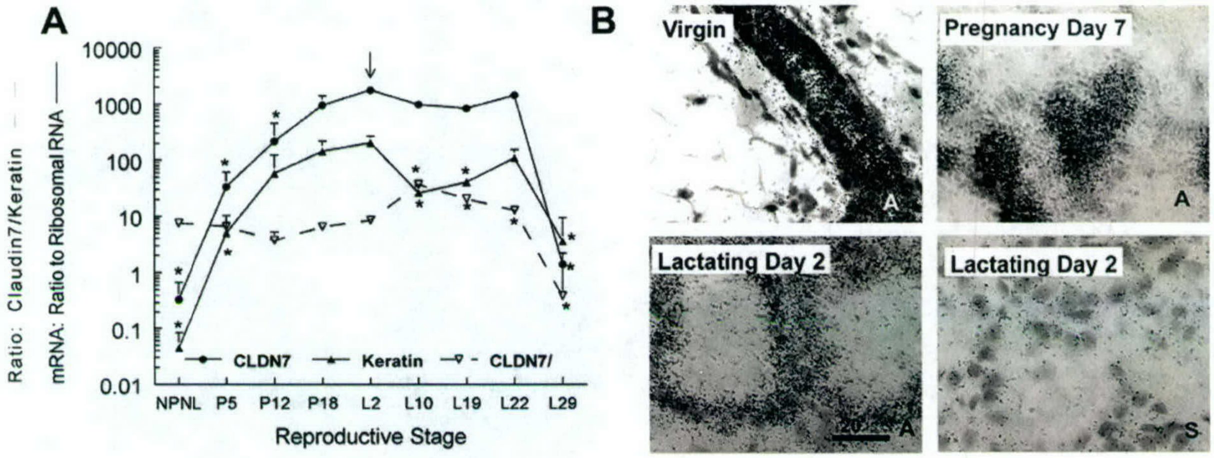


**Figure 1. Developmental expression of claudin 7 mRNA in the murine mammary gland.** A. Real time RT-PCR measurement of claudin 7 and keratin 19 from total mammary glands of non-pregnant non-lactating (NPNL) mice, mice at 5, 12, and 18 days of pregnancy (P5, P12, P18) and 2, 10, 19, 22 and 29 days post partum (L2, L10, L19, L22, L29). By L29 the pups have weaned themselves and mammary gland involution is nearly complete. Dotted line: Ratio of claudin-7 RNA to Keratin 19 at the same time points. Three mice analyzed at each time point. Bars are ± 1 s.e.m. Where no bar is apparent, the standard deviation falls within the symbol. Asterisks indicate points that differ significantly from values at L2, $p < 0.05$. B. *In situ* hybridization of claudin 7 probes to sections from virgin, pregnant and lactating mammary glands. Sections labeled A were hybridized to the antisense probe; the lower right hand section labeled S is the sense control. Bar is 20 microns.

**Figure 2.**

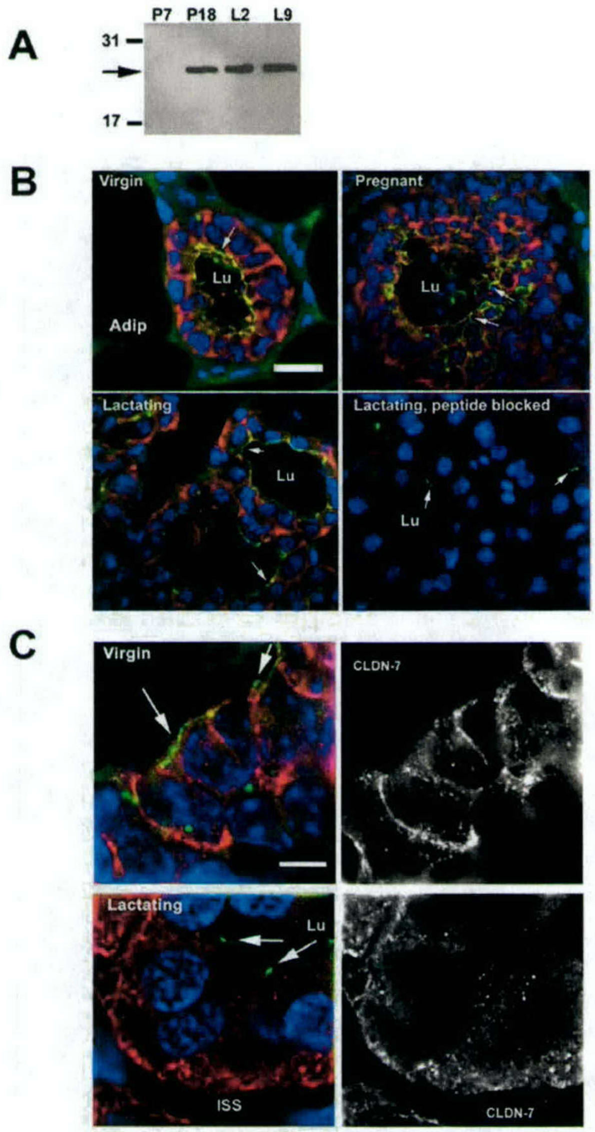
A
P7 P18 L2 L9
31
17
B
Virgin
Lu
Adip
Pregnant
Lu
Lactating
Lu
Lactating, peptide blocked
Lu
C
Virgin
CLDN-7
Lactating
Lu
ISS
CLDN-7

**Figure 2. Claudin 7 immunohistochemistry in the mammary gland.** A. Western blot on 50 μg protein from Pregnant day 7 and 18 (P7 and P18) and lactating days 2 and 9 (L2 and L9) mammary gland. B. Lower power views of epithelial structures from the developmental stages indicated with Fitc-labeled ZO-1 (green), Cy3-labeled claudin 7 (red), and DAPI-labeled nuclei (blue). Lu, lumen; Adip, Adipocyte. Lower right. Anticlaudin antibody blocked with claudin-7 peptide. Scale bar is 30 μm. C. High power views of claudin 7 localization. The right hand images show the merged views as in B. The left hand black and white images show only the corresponding claudin 7 stain demonstrating clearly the punctate nature of the claudin 7 distribution near the baso-lateral membranes; claudin 7 is excluded from both nuclei and tight junctions. The scale bar is equivalent to 10 μm. Arrows in all figures point to FITC-stained ZO-1 (green).

Figure 3.

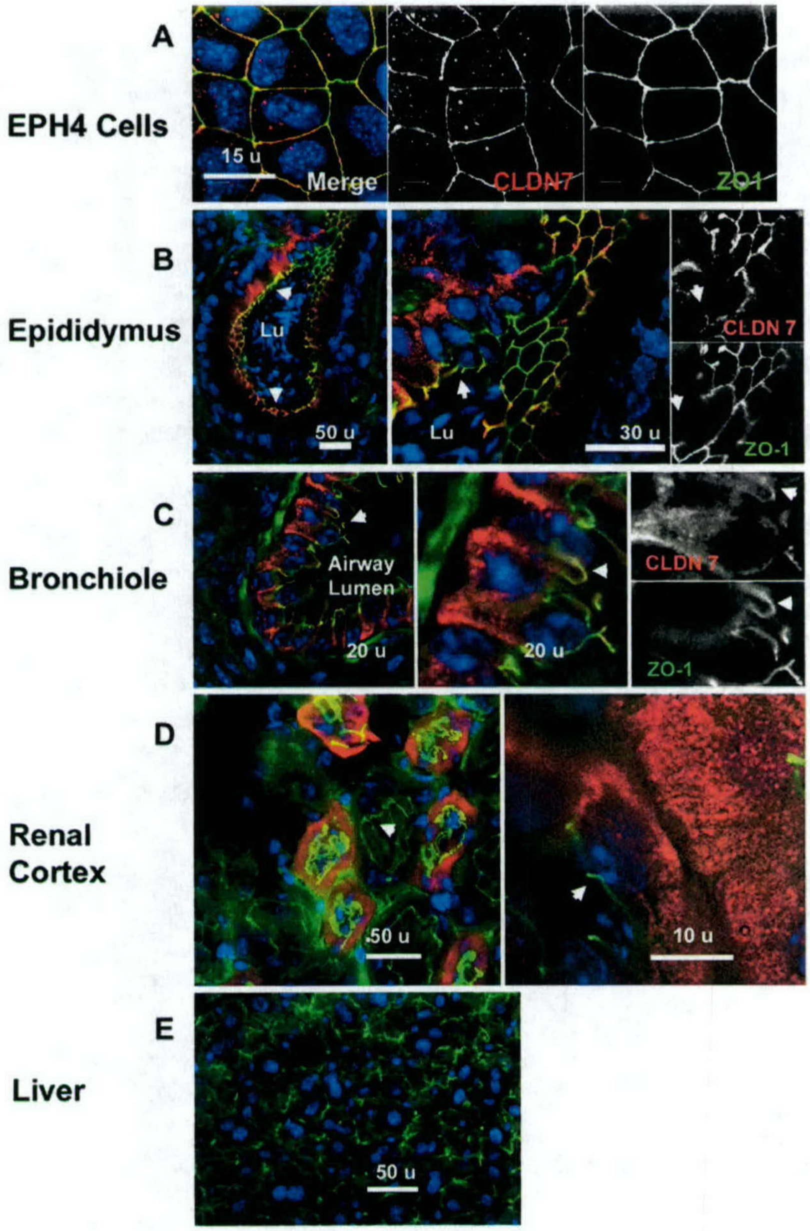
A
EPH4 Cells
15 u
Merge
CLDN7
ZO1
B
Epididymus
Lu
50 u
Lu
30 u
CLDN 7
ZO-1
C
Bronchiole
Airway Lumen
20 u
20 u
CLDN 7
ZO-1
D
Renal Cortex
50 u
10 u
E
Liver
50 u

**Figure 3. Immunolocalization of claudin-7 (Cy3, red) in cultured mammary epithelial cells, epididymus, lung, kidney, and liver.** ZO-1 is stained with FITC (green) to demonstrate location of the tight junctions. A. Eph4 cells. Claudin 7 is found with a slight patchy distribution at the tight junctions with ZO1. Vesicular stain can also be seen in the cytoplasm. B. Mouse Epidydimus. Claudin 7 stain colocalizes with ZO-1 in most, but not all tight junctions. LU, lumen. Small arrowheads in this and subsequent panels denote the apical border of the epithelium. C. Lung. The luminal cells of the bronchiole show heavy staining for claudin 7 in a punctate distribution at the basolateral surface of the cell; However, some claudin 7 colocalizes with ZO-1, see arrowheads in black and white inset. D. Kidney. Some of the tubules of the renal cortex stain as observed by Li et al. (2004). Claudin 7 shows a punctate distribution heaviest in the basal portion of the cells. E. Liver. Tight junctions at the renal canaliculi are stained with ZO-1. However, no stain for claudin 7 could be discerned. Magnification for each figure is indicated by the label above the scale bar.

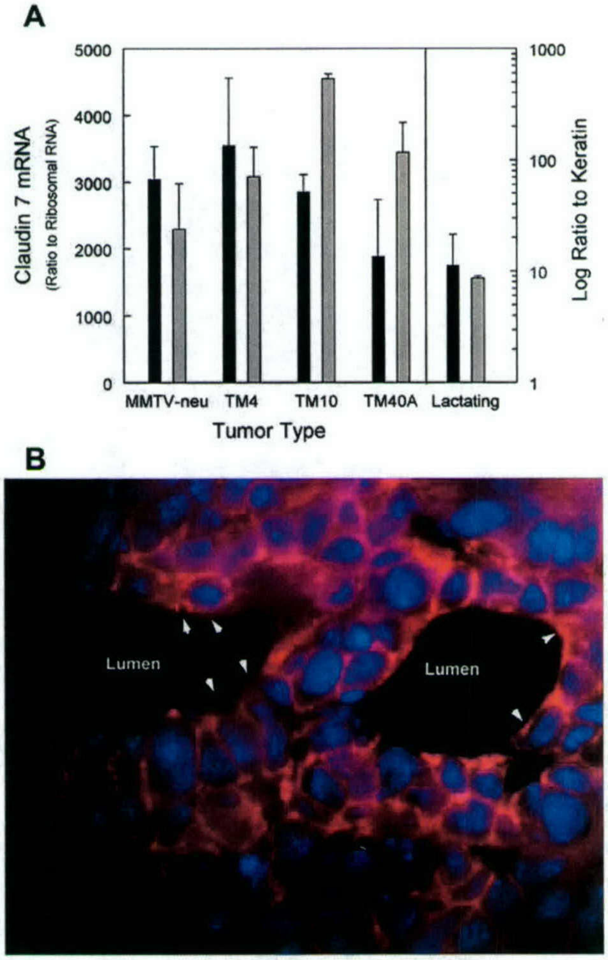


**Figure 4. Claudin 7 in mouse mammary tumors.** A. Black bars: mRNA levels by real time RT-PCR in four murine mammary tumors and the lactating gland, chosen for comparison because it, like the tumors, contains a large proportion of epithelial cells. All values are normalized to ribosomal RNA. Shaded bars: Ratio of claudin 7 mRNA to keratin 19 mRNA. B. Claudin 7 distribution in paraffin section of the transplantable tumor TM4. The stain is localized to the perimembrane cytoplasm in all cells and excluded from nuclei. The arrowheads mark the point where tight junction stain is expected if tight junctions were to form; however no ZO1 stain was detected in the section.

**Abstract ERA OF HOPE, Fall, 2002**

**EXPRESSION OF CLAUDIN 7 IN THE MOUSE MAMMARY EPITHELIUM**

Margaret C. Neville, Brigitte Blackman, Julia Foo, Valerie Sawicki
*DEPARTMENT OF PHYSIOLOGY AND BIOPHYSICS University of Colorado Health Sciences Center, Denver, CO 80220*

ABSTRACT: The claudins are a newly discovered family of proteins usually associated with tight junctions. Expression of one particular isoform of claudins, i.e., claudin 7, has been associated with breast tumors (Nacht M, Ferguson AT, Zhang W, et al. Cancer Res 1999;59:5464-5470). We have examined claudin-7 expression in mouse mammary tumors and during the development of the normal mammary gland using real time RT-PCR. We found that claudin 7 mRNA is increased more than 300 fold in early pregnancy, remains constant during lactation and is down-regulated at involution when compared to total RNA in the gland. However, the ratio of claudin 7 to an epithelial cell marker, cytokeratin-19 remains nearly constant through development suggesting that claudin-7 is a constitutive component of mammary epithelial cells. This hypothesis was confirmed by in situ localization of the mRNA to the mammary epithelium in mammary glands from virgin, pregnant and lactating mice. In contrast claudin 1 was most highly expressed in the virgin, claudin 3 was highly expressed during pregnancy and claudin 8 was significantly expressed only during lactation. Immunolocalization of claudin 7 in the normal mammary gland produced the surprising result that claudin-7 overlaps little with a marker of tight junctions, ZO-1, but rather appeared to be associated with both lateral and basal membranes of the cell. Claudin 1, on the other hand was localized to tight junctions in the epithelium of the virgin gland. These findings suggest that changes in the claudin complement play a significant role in the developmental changes of the mammary epithelium. In a panel of mouse mammary tumors the ratio of claudin-7 mRNA to cytokeratin mRNA was about 10 times higher than in the normal gland suggesting that the molecule might be used as a marker for breast cancer cells under certain circumstances. This project was supported by the Department of the Army Breast Cancer Research program awards DAMD 17-00-1-021 to NB and DAMD 17-98-1-8296

**Abstract presented at Breast Cancer Research Meeting, Sacramento, CA, October 2003**

**Expression of Claudin 7 in the mouse mammary epithelium and tumor models**

Margaret C. Neville, Brigitte Blackman, Julia Foo, Valerie Burns, Neal Beeman
Departments of Physiology and Biophysics, Cell and Developmental Biology and Obstetrics and Gynecology, UCHSC, Denver, CO. 80262,

The claudins are represented by a large family of membranes proteins usually associated with tight junctions. Expression of one particular isoform of claudins, i.e., claudin 7, has been associated with breast tumors (Nacht M, Ferguson AT, Zhang W, et al. Cancer Res 1999;59:5464-5470). We have examined claudin-7 expression in mouse mammary tumors and during the development of the normal mammary gland using real time RT-PCR. We found that claudin 7 mRNA is increased more than 300 fold in early pregnancy, remained constant during lactation and was down-regulated at involution when compared to total RNA in the gland. However, the ratio of claudin 7 to an epithelial cell marker, cytokeratin-19 remained nearly constant through development suggesting that claudin-7 is a constitutive component of mammary epithelial cells. This hypothesis was confirmed by in situ localization of the mRNA to the mammary epithelium in mammary glands from virgin, pregnant and lactating mice as well as the finding that it was expressed in all the mouse mammary tumors examined. In contrast claudin 3 was highly expressed during pregnancy and claudin 8 was significantly expressed only during lactation. Immunolocalization of claudin 7 in the normal mammary gland produced the surprising result that claudin-7 does not overlap with a marker of tight junctions, ZO-1, but rather appeared to be present in vesicles associated with both lateral and basal membranes of the cell. Claudin 1, on the other hand was localized to tight junctions in the epithelium of the virgin gland. Claudin 7 was localized to tight junctions in cultured mammary epithelial cells and the epididymus, but not the lung where it was also highly expressed. These findings suggest that claudin 7 is not involved in tight junctions in the mammary gland or lung but may be involved in interactions of these cells with the extracellular matrix. (Supported by DOD grant DAMD17-01-1-0211 to MCN).

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# SGI Year 2004 Annual Meeting

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**Presenting/Contact Author:** Margaret C. Neville, Ph.D.

**Department/Institution:** Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center

**Address:** 4200 E. Ninth Avenue

**City/State/Zip/Country:** Denver, CO, 80262, United States

**Phone:** 303 315 7343 **Fax:** 303 315 8110 **E-mail:** Peggy.neville@uchsc.edu

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**The presenting author is currently in training as an undergraduate or graduate student, a postdoctural fellow, medical student, resident or clinical fellow within a recognized academic program:** No

**Title:** EXPRESSION AND LOCALIZATION OF THE CLAUDINS IN MOUSE MAMMARY GLAND AND TUMOR MODELS

Margaret C. Neville, Ph.D. [1], Brigitte Blackman, Ph.D. [2]*, Julia Foo [2]*, Valerie Burns [1]* and Neal Beeman, M.S. [2]*. [1] Obstetrics and Gynecology, University of Colorado Health Sciences Center, Denver, CO, 80262 and [2] Physiology and Biophysics, University of Colorado Health Sciences Center, Denver, CO, 80262 .

Objective: To examine the hypothesis that claudins 3, 7, and 8 are associated with tight junction in the mammary gland.
Background: The claudins are represented by a large family of membranes proteins usually associated with tight junctions. Expression of one particular isoform of claudins, i.e., claudin 7, has been associated with breast tumors (Nacht M, Ferguson AT, Zhang W, et al. Cancer Res 1999;59:5464-5470).
Methods: We have examined claudin-7 expression in mouse mammary tumors and during the development of the normal mammary gland using real time RT-PCR and immunolocalization. Results: We found that claudin 7 mRNA is increased more than 300 fold in early pregnancy, remained constant during lactation and was down-regulated at involution when compared to total RNA

in the gland. However, the ratio of claudin 7 to an epithelial cell marker, cytokeratin-19 remained nearly constant through development suggesting that claudin-7 is a constitutive component of mammary epithelial cells. This hypothesis was confirmed by in situ localization of the mRNA to the mammary epithelium in mammary glands from virgin, pregnant and lactating mice as well as the finding that it was expressed in all the mouse mammary tumors examined. In contrast claudin 3 was highly expressed during pregnancy and claudin 8 was significantly expressed only during lactation. Immunolocalization of claudin 7 in the normal mammary gland produced the surprising result that claudin-7 does not overlap with a marker of tight junctions, ZO-1, but rather appeared to be present in vesicles associated with both lateral and basal membranes of the cell. (Figure 1) Claudin 1, on the other hand was localized to tight junctions in the epithelium of the virgin gland. Claudin 7 was localized to tight junctions in cultured mammary epithelial cells and the epididymus, but not the lung where it was also highly expressed. Conclusions: These findings suggest that claudin 7 is not involved in tight junctions in the mammary gland or lung but may be involved in interactions of these cells with the extracellular matrix. (Supported by DOD grant DAMD17-01-1-0211 to MCN).

Signature of Presenting/Contact Author:

Margaret C. Neville

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